

REMARKS

Applicants respectfully request reconsideration of the present application in view of the reasons that follow.

I. Claim Status

No claims are currently amended. Claims 4, 6, 8-13 and 15-25 are now pending in this application.

II. Claim Rejections under 35 U. S. C. § 112

Claims 4, 6, 8-13 and 15-25 are rejected under 35 U.S.C. 112, first paragraph for not providing enablement for a method of inhibiting angiogenesis in a subject in need thereof. Applicants respectfully traverse.

The Office Action opines that (1) “the data is confusing in that it is not clear what Applicants are measuring, particularly with regards to Experiment 2, where the results appear to be the same as the TSP-1 itself;” (2) “the description of both experiments is lacking details, like how the TSP-1 activity was measured in the luciferase assay, nor is there a control using a known TSP-1 inducer to demonstrate activity;” (3) “Applicants do not provide any indication of how they have made each extract or what the extracts are;” (4) “there are no experiments conducted to demonstrate a correlation between TSP-1 activity or luciferase and inhibition of wrinkles caused by any mechanism, particularly photoaging;” and (5) “no working examples are provided with regard to a method of inhibiting angiogenesis in a subject in need thereof [with regard to claims 4, 12 and 19]” and “the method of inhibiting angiogenesis inhibits wrinkles caused by photoaging of the skin of said subject [with regard to claim 12]”, and thus alleges that “the quantity of experimentation necessary to carry out the claimed invention is high.” Applicants respectfully disagree for at least the reasons that follow.

A. Objections Of Items (1)-(2) Are Improper.

In contrast to the allegations made in the Office Action, the Specification explicitly discloses that, in Experiment 1, luciferase activity was measured with Promega Luciferase Assay System (*see* the Specification, Page 10, Lines 26-29). Applicants respectfully submit that the method of measuring luciferase activity by Promega Luciferase Assay System is well known in the field. Support for this statement can be found in Exhibit A (Promega's "Technical Bulletin No.281"), which provides detailed instructions for using the assay system.

The Office Action also alleges that there is no control in Experiment 2. Applicants respectfully disagree. The object of Experiment 2 is the screening of agents capable of exhibiting TSP-1 function. Therefore, TSP-1 itself was naturally used a positive control in Experiment 2. Negative controls (e.g., Medium and DMSO) were also provided in Experiment 2. Regents demonstrating cell death induction to an extent similar to TSP-1 itself (i.e., the positive control) are deemed to have TSP-1 function.

Further, the Specification explicitly teaches that the detection of the cell death was carried out by using an Apoptag Plus Fluorescein In Situ Apoptosis Detection Kit, followed by calculating the proportion of the number of dead cells in the group (to which the agent had been added) relative to that of the control group. The method of measuring cell death using Apoptag Plus Fluorescein In Situ Apoptosis Detection Kit is well known in the field. For example, Exhibit B (Jimenez et al., *Nature Medicine*, V. 6 (1), 41, 2000) describes using the kit to measure the apoptosis activity of TSP-1 (see Exhibit B, page 46, right column, Section "Apoptosis Assay").

Thus, Applicants respectfully submit that the experimental details are readily accessible to the public and that Experiment 2 does include both positive and negative controls, in contrast to what the Office Action alleges.

B. Objections Of Item (3) Is Improper.

The Office Action objects that “Applicants do no provide any indication of how they have made each extract or what the extracts are.” Applicants respectfully traverse for the reasons that follow.

First, the main components of the extracts are described in the Specification on pages 6-7. Second, the extracts used for the working example are commercially available. Further, the source of the extracts used for the working example are provided in the Specification (e.g., from Ichimaru Pharcos Co. Ltd.). With the above information provided in the Specification, Applicants respectfully submit that enough detail has been provided to enable an ordinary skill in the art to carry out the invention without undue experimentation.

C. Objections Of Items (4)-(5) Are Improper.

Independent claims 4, 12 and 19 and dependent claims thereof recite “a method of inhibiting angiogenesis in a subject in need thereof.”

As explained in the Specification (*see* Specification, Page 5, Lines 1-15), inhibiting angiogenesis with TSP-1 activity (luciferase activity) is highly correlated with the induction of apoptosis of endothelial cells. Support for this statement can also be found in Exhibit B, which explicitly describes that TSP-1 inhibits angiogenesis by inducing apoptosis in endothelial cells (*see* Exhibit B, the last paragraph of page 46, left column), and in Exhibit C (Yano et al., *J. Invest. Dermatol.* 118: 800-855, 2002) and Exhibit D (Detmar, WO 02/083088) disclosing that TSP-1 prevents UV-B induced skin damage via its angiogenesis inhibitory activity.

Thus, the limitation of “a method of inhibiting angiogenesis in a subject in need thereof” recited in claims 4, 12 and 19 are well supported and enabled by the working example of Experiments 1 and 2, which clearly show respectively the TSP-1 activity (luciferase activity) and the induction of apoptosis of endothelial cells by the claimed extracts.

Further, the reagents that inhibit angiogenesis can effectively prevent or inhibit aging (*see* Page 2, Lines 19-25). Thus, the limitation of “wherein the method of inhibiting

angiogenesis inhibits wrinkles caused by photoaging of the skin of said subject,” as recited in claim 12 is also enabled.

II. Claim Rejections under 35 U. S. C. §§ 102&103

Claims 4 and 12 are rejected under 35 U.S.C. 102(b) as being anticipated by Uehara et al. (JP 2000-119156, hereinafter “Uehara”). Claims 4, 11, 12 and 18 remain rejected under 35 U.S.C. 102(b) as being anticipated by Garlen et al. (US 4,707,354, hereinafter “Garlen”). Claims 4, 6, 8-13 and 15-25 are rejected under 35 U.S.C. 103(a) as being unpatentable over Sei et al. (JP 2002-128651, hereinafter “Sei”), in view of Andre-Jean et al. (JP 07-145067, hereinafter “Andre-Jean”).

Independent claims 4, 12 and 19 recite “a method of inhibiting angiogenesis in a subject in need thereof.” The preamble, “in need thereof”, is not merely a statement of effect that may or may not be desired or appreciated, but rather is a statement of the intentional purpose for which the method must be performed. *Jansen v. Rexall Sundown, Inc.*, 342 F.3d 1329, 1333-34, 68 USPQ2d 1154, 1158 (Fed. Cir. 2003).

The Office Action acknowledges that the rejections based on Uehara, Garlen, Sei and Andre-Jean were made without considering the limitation of “inhibiting angiogenesis in a subject in need thereof” on the alleged ground that the claimed limitation of “inhibiting angiogenesis in a subject in need thereof” is not enabled. However, as explained above, “a method of inhibiting angiogenesis in a subject in need thereof” recited in independent claims 4, 12 and 19 are actually enabled, in contrast to what is alleged in the Office Action, and thus should be considered in evaluating the patentability of the claims.

Also, as explained in the previous response filed on September 8, 2009, the references cited by the Office Action are silent in respect of inhibiting angiogenesis. None of these references teaches that any of the disclosed formulations or crude drugs are capable of inhibiting angiogenesis. Accordingly, Applicants believe that the claimed method of claim 4, 12 and 19 are not anticipated by, or obvious over, the cited references.

Claims 6 and 8-11 depend from claim 4, and thus are patentable for at least the same reasons as claim 4. Claims 13 and 15-18 depend from claim 12, and thus are patentable for at

least the same reasons as claim 12. Claims 20-25 depend from claim 19, and thus are patentable for at least the same reasons as claim 19.

For at least the above reasons, Applicants respectfully request a withdrawal of the sections 102 and 103 rejections.

III. Conclusion

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing or a credit card payment form being unsigned, providing incorrect information resulting in a rejected credit card transaction, or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorize payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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EXHIBIT A

Luciferase Assay System



Technical Bulletin No. 281

INSTRUCTIONS FOR USE OF PRODUCTS E1483, E1500, E1501, E1531, E4030, E4530 AND E4550.

All technical literature is available on the Internet at www.promega.com

Please visit the web site to verify that you are using the most current version of this Technical Bulletin.

I. Description	1
II. Product Components	3
III. Preparations Prior to Performing the Luciferase Assay	4
A. Determining the Linear Range of Light Detection.....	4
B. Luciferase Assay Reagent Preparation	4
C. Lysis Buffers	4
D. Protocol for Preparing Cell Lysates	5
E. Protocol for Plant and Bacterial Cell Lysates and Tissue Homogenates.....	5
IV. Luciferase Assay Protocol.....	5
A. Protocol for Manual Luminometers.....	6
B. Protocol for Single-Tube Luminometers with Injectors.....	6
C. Protocol for Plate Reading Luminometers	6
V. General Considerations	6
A. Optimization of Light Intensity	6
B. Instrumentation.....	7
C. Firefly Luciferase Reporter Vectors	8
VI. Composition of Buffers and Solutions	9
VII. Related Products	10
VIII. References	11
<i>Experienced User's Protocol</i>	13

I. Description

Genetic reporters are used commonly in cell biology to study gene expression and other cellular events coupled to gene expression, such as receptor activity, intracellular signal transduction, mRNA processing, protein folding and protein-protein interactions (1,2). Firefly luciferase is widely used as a reporter for the following reasons:

- Reporter activity is available immediately upon translation since the protein does not require post-translational processing (3,4).
- The assay is very sensitive because its light production has the highest quantum efficiency known for any chemiluminescent reaction (5) and no background luminescence is found in the host cells or the assay chemistry.
- The assay is rapid, requiring only a few seconds per sample.

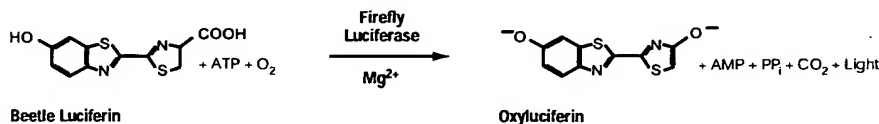


Figure 1. Bioluminescent reaction catalyzed by firefly luciferase.





Promega's Luciferase Assay System^(a,b) is substantially improved over conventional assay methods in both sensitivity and simplicity (2,6–8). Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase, a monomeric 61kDa protein, catalyzes luciferin oxidation using ATP•Mg²⁺ as a cosubstrate (Figure 1). In the conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined. Promega's Luciferase Assay System incorporates coenzyme A (CoA) for improved kinetics (9), allowing greater enzymatic turnover resulting in increased light intensity that is nearly constant for at least 1 minute (Figure 2). The Luciferase Assay System yields linear results over at least eight orders of magnitude. Less than 10⁻²⁰ moles of luciferase have been detected under optimal conditions (2). Generally, 100-fold greater sensitivity can be achieved over the chloramphenicol acetyltransferase (CAT) assay (1).

Promega's Luciferase Assay System was developed for reporter quantitation in mammalian cells. The Luciferase Assay System (Cat.# E1500), provided with Cell Culture Lysis Reagent (CCLR); can also be used for reporter quantitation in plant and bacterial cells (see Section III.E); however, the Luciferase Assay System with Reporter Lysis Buffer (Cat.# E4030) is not suitable for these applications.

The Luciferase Reporter 1000 Assay System (Cat. #E4550) was designed to meet the needs of users who perform a large number of assays, particularly in 96-well plates. The system contains sufficient reagents to perform 1,000 luciferase assays (100µl per assay). For users working with transformed cells, a cell lysis buffer will be needed for sample preparation prior to luciferase measurement (see Section III). The lysis buffer must be purchased separately.

The Luciferase Assay System is generally used with a lysis buffer and Luciferase Assay Reagent. Luciferase Assay Reagent and its preparation are described in Section III.B. The three lysis buffers are described in Section III.C and Table 1 recommends the appropriate lysis buffer for use with a particular cell type.

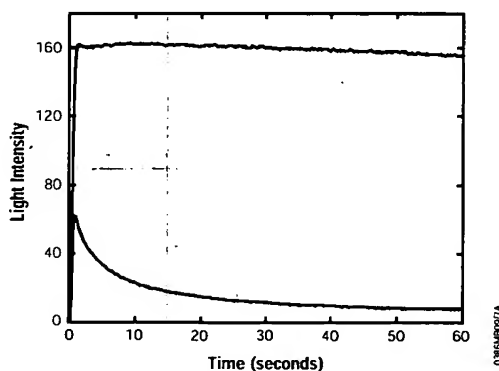


Figure 2. Comparison of Promega's Luciferase Assay System to the conventional luciferase assay method. NIH3T3 cells expressing the luciferase gene from Rous sarcoma virus were lysed with 1X Cell Culture Lysis Reagent 48 hours after infection. The shaded area represents the light typically lost in measurements where the cell lysate is mixed with substrate prior to light detection (e.g., scintillation counting). In the conventional assay, this is 50% of the total luminescence in a 1-minute measurement.

II. Product Components

Product	Size	Cat.#
Luciferase Assay System ^(a,b)	100 assays	E1500

Each system contains sufficient reagents for 100 standard assays. Includes:

- 1 vial Luciferase Assay Substrate (lyophilized)
- 10ml Luciferase Assay Buffer
- 30ml Luciferase Cell Culture Lysis Reagent, 5X
- 1 Protocol

Product	Size	Cat.#
Luciferase Assay System 10-Pack ^(a,b)	1,000 assays	E1501

Contains sufficient reagents for 1,000 standard assays. Includes:

- 10 vials Luciferase Assay Substrate (lyophilized)
- 10 × 10ml Luciferase Assay Buffer
- 1 Protocol

Product	Size	Cat.#
Luciferase Assay System with Reporter Lysis Buffer ^(a,b)	100 assays	E4030

Each system contains sufficient reagents for 100 standard assays. Includes:

- 1 vial Luciferase Assay Substrate (lyophilized)
- 10ml Luciferase Assay Buffer
- 30ml Reporter Lysis 5X Buffer
- 1 Protocol

Product	Size	Cat.#
Luciferase Assay System Freezer Pack ^(a,b)	1,000 assays	E4530

Each system contains sufficient reagents for 1,000 standard assays. Includes:

- 10 vials Luciferase Assay Substrate (lyophilized)
- 10 × 10ml Luciferase Assay Buffer
- 10 vials Reporter Lysis 5X Buffer (30ml/vial)
- 10 Protocols

Product	Size	Cat.#
Luciferase Reporter 1000 Assay System ^(a,b)	1,000 assays	E4550

Each system contains sufficient reagents for 1,000 standard assays. Includes:

- 1 vial Luciferase Assay Substrate (lyophilized)
- 105ml Luciferase Assay Buffer
- 1 Protocol

Product	Size	Cat.#
Luciferase Assay Reagent ^(a,b)	1,000 assays	E1483

Contains sufficient reagent for 1,000 standard assays. Includes:

- 100ml Luciferase Assay Reagent
- 1 Protocol

Storage and Stability: Luciferase Assay Reagent can be purchased ready to use (Cat.# E1483) or prepared by reconstituting Luciferase Assay Substrate with Luciferase Assay Buffer. Luciferase Assay Reagent should be stored in aliquots and is stable at -20°C for up to 1 month, or at -70°C for up to 1 year after reconstitution or initial use. After preparation and freezing, the Luciferase Assay Reagent should be mixed well before use. Nonreconstituted system components may be stored at -20°C for 1 year. Store Luciferase Assay Substrate in the dark. Reporter Lysis Buffer may be stored at room temperature and should be stored away from direct sunlight. Cell Culture Lysis Reagent should be stored at -20°C.



Do not store
the Luciferase Assay
Reagent with dry ice.



Do not thaw
the Luciferase Assay
Reagent at tempera-
tures above 25°C.



Note: Luciferase Assay Reagent is also available premixed (Cat.# E1483).



For applications involving the coexpression of firefly luciferase with a second reporter gene, we recommend preparing cell lysates with either RLB or PLB.

III. Preparations Prior to Performing the Luciferase Assay

Before beginning a luciferase assay for the first time, prepare the Luciferase Assay Reagent (Section III.B) and the lysis buffer (Section III.C–D). Important light detection considerations are noted in Section III.A. In addition, Section V provides information on optimizing light intensity and choice of light detection instrumentation.

A. Determining the Linear Range of Light Detection

It is important to determine the linear range of light detection for your luminometer before performing an experiment, because luminometers can experience signal saturation at high light intensities. To produce a standard curve of light units versus relative enzyme concentration, make serial dilutions of luciferase (either purified luciferase or cell culture lysate) in any 1X lysis buffer supplemented with 1mg/ml BSA. The addition of BSA is necessary to ensure that luciferase is not lost from solution by adsorption. Recombinant firefly luciferase is available from Promega (QuantiLum® Recombinant Luciferase^(c), Cat.# E1701).

B. Luciferase Assay Reagent Preparation

To prepare the **Luciferase Assay Reagent**, add **Luciferase Assay Buffer** (105ml for Cat.# E4550; 10ml for other systems) to the vial containing the lyophilized **Luciferase Assay Substrate**. Avoid exposure of the Luciferase Assay Reagent to multiple freeze-thaw cycles by dispensing the reconstituted reagent into working aliquots. Store any unused Luciferase Assay Reagent at –70°C. Equilibrate Luciferase Assay Reagent to room temperature before each use. Each reaction requires 100µl of the Luciferase Assay Reagent to initiate enzyme activity.

C. Lysis Buffers

Promega has three lysis buffers that can be used to prepare cell lysates containing luciferase (see Table 1). Luciferase Cell Culture Lysis Reagent (CCLR) provides efficient lysis within minutes. Reporter Lysis Buffer (RLB) is a mild lysis agent and requires a single freeze-thaw cycle to achieve complete cell lysis. Passive Lysis Buffer (PLB; Cat.# E1941) will passively lyse cells without the requirement of a freeze-thaw cycle. However, lysis efficiency is dependent upon the cell type and needs to be determined for those cells that are resistant to passive lysis. PLB contains an anti-foam agent, which prevents excessive bubbling of the sample when the reagent is delivered with force by an automated dispenser. The absence of bubble formation may result in more consistent detection of light output and prevents instrument contamination.

Table 1. Recommended Lysis Buffers for Various Sample Types.

Sample/Cell Type	Lysis Buffer
adherent mammalian cells	CCLR, RLB, PLB
nonadherent mammalian cells	CCLR, RLB, PLB
bacterial cells ^{a,b}	CCLR
plant cells ^b	CCLR
tissue homogenates	CCLR, RLB

^aSection VI contains information on the CCLR formulation (lysis mix) recommended for bacterial cell lysis.

^bRLB has not been qualified for use with plant or bacterial cells.

D. Protocol for Preparing Cell Lysates

1. Add 4 volumes of water to 1 volume of 5X lysis buffer. Equilibrate 1X lysis buffer to room temperature before use.
2. Carefully remove the growth medium from cells to be assayed. Rinse cells with PBS (see Section VI), being careful to not dislodge attached cells. Remove as much of the PBS rinse as possible.
3. Add enough 1X lysis buffer (CCLR, RLB or PLB) to cover the cells (e.g., 400 μ l/60mm culture dish, 900 μ l/100mm culture dish or 20 μ l per well of a 96-well plate). If using RLB, perform a single freeze-thaw to ensure complete lysis. **For 96-well plates, proceed to Section IV. For culture dishes, continue to Step 4.**
4. Rock culture dishes several times to ensure complete coverage of the cells with lysis buffer. Scrape attached cells from the dish. Transfer cells and all liquid to a microcentrifuge tube. Place the tube on ice.
5. Vortex the microcentrifuge tube 10–15 seconds, then centrifuge at 12,000 $\times g$ for 15 seconds (at room temperature) or up to 2 minutes (at 4°C). Transfer the supernatant to a new tube.
6. Store the supernatant/cell lysate at –70°C or proceed to Section IV.

E. Protocol for Plant and Bacterial Cell Lysates and Tissue Homogenates

1. For plant tissue, quick-freeze in liquid nitrogen, grind the frozen tissue to a powder and resuspend at room temperature in 1X CCLR with further homogenization. Remove the debris after cell lysis by a brief centrifugation. Assay the supernatant using standard assay conditions (Section IV).
2. For bacteria, mix 40 μ l of nontransformed cells (carrier cells) with 50 μ l of a transformed culture. Add 10 μ l of 1M K₂HPO₄ (pH 7.8), 20mM EDTA. Quick-freeze the mixture on dry ice, then bring the cells to room temperature by placing the tube in a room temperature water bath. Add 300 μ l of freshly prepared lysis mix (Section VI). Mix and incubate the cells for 10 minutes at room temperature. To assay the lysate, proceed to Section IV.
3. A protocol for the use of tissue homogenates with the Luciferase Assay System can be found in reference 10.

IV. Luciferase Assay Protocol

Material to Be Supplied by the User

- opaque multiwell plates or luminometer tubes

The following procedures (Section IV.A–C) are optimized for mammalian cells grown in culture and may also be used with bacterial and plant cell lysates or tissue homogenates, as prepared in Section III.E. The Luciferase Assay System may be used with manual luminometers (those without reagent injectors) or with luminometers that have injectors (either single tube or 96-well plate types).

Note: An Experienced User's Protocol can be found at the end of this Technical Bulletin.



The Luciferase Assay Reagent and samples should be at ambient temperature prior to performing a luciferase assay (see Section V.A).



Note: When using shorter assay times, validate the luminometer over that time period to ensure that readings are taken at a flat portion of the signal curve.

A. Protocol for Manual Luminometers

1. Dispense 100 μ l of the Luciferase Assay Reagent into luminometer tubes, one tube per sample.
2. Program the luminometer to perform a 2-second measurement delay followed by a 10-second measurement read for luciferase activity. The read time may be shortened if sufficient light is produced.
3. Add 20 μ l of cell lysate to a luminometer tube containing the Luciferase Assay Reagent. Mix by pipetting 2–3 times or vortex briefly.
4. Place the tube in the luminometer and initiate reading.
5. If the luminometer is not connected to a printer or computer, record the reading.

B. Protocol for Single-Tube Luminometers with Injectors

1. Prime the luminometer injector at least three times with Luciferase Assay Reagent or as recommended in the owner's manual.
2. Dispense 20 μ l of cell lysate or test sample into a luminometer tube.
3. Program the luminometer to perform a 2-second measurement delay followed by a 10-second measurement read for luciferase activity. The read time may be decreased if sufficient light is produced.
4. Place the tube in the luminometer and initiate reading by injecting 100 μ l of Luciferase Assay Reagent into the tube.
5. If the luminometer is not connected to a printer or computer, record the reading.

C. Protocol for Plate Reading Luminometers

1. Program the luminometer for the appropriate delay and measurement times.
2. Place the plate, containing 20 μ l of cell lysate per well, into the luminometer with injector. The injector adds 100 μ l of Luciferase Assay Reagent per well, then the well is read immediately. The plate is advanced to the next well for a repeat of the inject-then-read process.
3. Measure the light produced for a period of 10 seconds. The light intensity of the reaction is nearly constant for about 1 minute and then decays slowly, with a half-life of approximately 10 minutes. The typical delay time is 2 seconds and the typical read time is 10 seconds. The assay time may be shortened significantly to decrease the total read time if sufficient light is produced. **For example**, the total read time for all samples in a 96-well plate can be less than 5 minutes.

V. General Considerations

A. Optimization of Light Intensity

Light intensity is a measure of the rate of catalysis by luciferase and is therefore dependent upon temperature. The optimum temperature for luciferase activity is approximately room temperature (20–25°C). It is important that the Luciferase Assay Reagent be fully equilibrated to room temperature before beginning measurements. To ensure temperature equilibration, place a thawed aliquot of the Luciferase Assay Reagent in a sealed tube into a water bath maintained at ambient temperature, and equilibrate for at least 30 minutes.

The sample to be assayed should also be at ambient temperature. Generally, luciferase activity is stable for several hours at room temperature in 1X Luciferase Cell Culture Lysis Reagent (Cat.# E1531), Reporter Lysis Buffer (Cat.# E3971) or Passive Lysis Buffer (Cat.# E1941). If specific circumstances make ambient temperature unacceptable, the sample may be left on ice for up to 12 hours. Assay of a cold sample (0–4°C) using standard assay volumes (see Section IV) will result in a 5–10% decrease in enzyme activity.

Note: Cell lysates prepared using CCLR (Luciferase Cell Culture Lysis Reagent) will not yield optimal results when assaying for CAT, β -galactosidase or *Renilla* luciferase coreporter activities. CAT is partially inhibited by the Triton® X-100 component of CCLR (11). Although β -galactosidase is not directly inhibited by the high detergent concentration of CCLR, a precipitate may form upon mixing β -Galactosidase Assay Buffer with cell lysates prepared using this lysis buffer. The composition of CCLR and RLB significantly inhibits *Renilla* luciferase activity, and also contributes excessive levels of coelenterazine auto-luminescence (12). Furthermore, the high concentration of detergent and dithiothreitol (DTT) in CCLR precludes the use of most protein determination assays to quantify total protein in cell lysates prepared with CCLR.

B. Instrumentation

Either a luminometer or a scintillation counter can be used for quantitation with the Luciferase Assay System. (There is usually insufficient light output for qualitative visual detection.) A luminometer can measure as little as 10⁻²⁰ moles (0.001pg) of luciferase, whereas a scintillation counter typically has a less sensitive detection limit. However, the limits of sensitivity may vary depending upon the particular instrument used. The assay should be linear in some portion of the detection range of the instrument. Please consult your instrument operator's manual for general operating instructions.

Luminometers

The most convenient method for performing a large number of luciferase assays is to use a luminometer capable of processing a multiwell plate. The light intensity of the assay and the effective linear range is proportional to luciferase concentrations in the range of 10⁻²⁰ to 10⁻¹³ moles. However, the limits of sensitivity may vary, depending upon the particular instrument used. The limits should be verified on each instrument before analysis of experimental samples (see Section III.A).

Scintillation Counters

Ideally, the coincidence circuit of the scintillation counter should be turned off. Usually, this is achieved through an option of the programming menu or by a switch within the instrument. If the circuit cannot be turned off, a linear relationship between luciferase concentration and cpm still can be produced by calculating the square root of measured counts per minute (cpm) minus background cpm (i.e., [sample – background]^{1/2}). To measure background cpm, add Luciferase Assay Reagent to lysis buffer without cells or to a lysate of nontransfected cells.

The sample may be placed directly in the scintillation vial if it completely covers the bottom of the vial (clear or translucent vials are acceptable). Do not add scintillant, because it will inactivate luciferase. Alternatively, place the sample in a



For applications involving the coexpression of firefly luciferase with a second reporter gene, we recommend preparing cell lysates with either RLB or PLB.

microcentrifuge tube, and then place the tube in the scintillation vial. To ensure consistency when working with multiple samples, place each microcentrifuge tube at the same relative position within the scintillation vial.

For consistency in measuring luciferase activity, use the scintillation counter in manual mode. Initiate each sample reaction immediately before measurement and read the samples one at a time. Because the enzymatic reaction produces light at all wavelengths, read the samples with all channels open (open window). To reduce background counts, it may be necessary to wait 10–30 seconds before counting. Read individual samples for 1–5 minutes.

C. Firefly Luciferase Reporter Vectors

The pGL3 Luciferase Reporter Vectors^(c,d) contain the cDNA encoding luciferase (*luc+*) cloned from the North American firefly (*Photinus pyralis*) and a vector backbone that has been designed to provide enhanced reporter gene expression. Modifications that distinguish *luc+* from the native luciferase gene (*luc*) fall into four general categories: i) the C-terminal tripeptide is substituted to eliminate peroxisome targeting of the expressed reporter enzyme; ii) codon usage is improved for increased expression in plant and animal cells; iii) two potential sites of N-glycosylation are modified; and iv) several DNA sequence changes are incorporated to disrupt extended palindromes, remove internal restriction sites and eliminate consensus sequences that may be recognized by genetic regulatory binding proteins. Changes in luciferase reporter activity directly correlate to the transcriptional activity of the cloned regulatory element when expressed in transfected cells. These modifications help to ensure that the luciferase reporter gene does not contribute spurious transcriptional signals. Further details on these modifications are provided in the *pGL3 Luciferase Reporter Vectors Technical Manual* #TM033.

In addition to changes made to the luciferase gene, four major modifications were incorporated into the vector backbone of the pGL3 family of luciferase vectors: i) the SV40 early poly(A) signal is replaced with the SV40 late poly(A) signal for improved RNA processing (13); ii) a synthetic poly(A) and transcription pause site is positioned upstream of the multiple cloning region to terminate spurious transcription, which may initiate within the vector backbone (14); iii) the small t intron is removed to eliminate cryptic splicing, resulting in greater reporter gene expression (15); and iv) the Kozak consensus sequence is added to increase the efficiency of luciferase translation initiation (16).

The modifications embodied in the pGL3 Vector family provide greater flexibility in performing genetic manipulations, minimal relative background activity and luciferase expression levels that are dramatically higher than previously obtained with the pGL2 Vectors^(c). Using the pGL3 Vectors, it is now possible to obtain measurable luciferase expression in cell types that are difficult to transfect, when studying weak promoter elements or when performing *in vivo* luminescence measurements. It is important to recognize that absolute light unit values and relative expression profiles of reporter vectors will vary between cell types. We recommend that the appropriate control vector always be included in experiments utilizing genetic reporter systems.

The pGL3 Vector family comprises four types of improved firefly luciferase vectors, the pGL3-Basic Vector, the pGL3-Promoter Vector, the pGL3-Enhancer Vector and the pGL3-Control Vector. The pGL3-Basic Vector (Cat.# E1751) lacks eukaryotic promoter and enhancer elements. The strategic placement of unique

restriction enzyme sites within this vector provides maximum flexibility in cloning and the ability to further manipulate putative genetic regulatory sequences. Expression of luciferase activity in cells transfected with the pGL3-Basic Vector is dependent upon the insertion of a functional promoter upstream of *luc+*. In addition, desired enhancer elements may be inserted at positions that flank the immediate promoter sequence or may be positioned downstream of *luc+*. The pGL3-Promoter Vector (Cat.# E1761) contains an SV40 promoter upstream of *luc+*. Genomic DNA fragments containing putative enhancer elements can be inserted in either orientation, upstream or downstream from the SV40 promoter/*luc+* transcriptional unit. The pGL3-Enhancer Vector (Cat.# E1771) contains an SV40 enhancer downstream of the *luc+* reporter gene. This allows verification of functional promoter/*luc+* junctions when testing putative promoter sequences. The presence of an enhancer will, in many cases, provide increased transcriptional activity of cloned promoter elements. The pGL3-Control Vector (Cat.# E1741) contains both SV40 promoter and enhancer sequences, resulting in strong expression of luciferase activity in many types of eukaryotic cells. This plasmid is useful for general monitoring of transfection efficiency.

VI. Composition of Buffers and Solutions

PBS buffer (Mg²⁺- and Ca²⁺-free)

137mM	NaCl
2.7mM	KCl
4.3mM	Na ₂ HPO ₄
1.4mM	KH ₂ PO ₄

The final pH should be 7.3.

lysozyme (5mg/ml)

Add 1 volume of 1M K₂HPO₄ (pH 7.8), 20mM EDTA to 9 volumes of water. Add lysozyme to a final concentration of 5mg/ml. Vortex until the lysozyme dissolves. Prepare fresh for each use.

Luciferase Cell Culture Lysis Reagent, 1X

25mM	Tris-phosphate (pH 7.8)
2mM	DTT
2mM	1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
10%	glycerol
1%	Triton® X-100

lysis mix

1X	CCLR
1.25mg/ml	lysozyme
2.5mg/ml	BSA

Add water to desired volume. Prepare fresh for each use.



VII. Related Products

Luciferase Assay Systems and Reagents

Product	Size	Cat.#
Reporter Lysis Buffer, 5X	30ml	E3971
Luciferase Cell Culture Lysis Reagent, 5X	30ml	E1531
Passive Lysis Buffer, 5X	30ml	E1941
Beetle Luciferin, Potassium Salt	5mg	E1601
	50mg	E1602
	250mg	E1603
QuantiLum® Recombinant Luciferase(c)	1mg	E1701
	5mg	E1702

Product	Size	Cat.#
Steady-Glo™ Luciferase Assay System(a,b)	10ml	E2510
	100ml	E2520
	10 × 100ml	E2550
Bright-Glo™ Luciferase Assay System(a,b)	10ml	E2610
	100ml	E2620
	10 × 100ml	E2650

Dual-Luciferase® Reporter Assay Systems

Product	Size	Cat.#
Dual-Luciferase® Reporter Assay System(a,b,e)	100 assays	E1910
Dual-Luciferase® Reporter Assay System 10-Pack(a,b,e)	1,000 assays	E1960
Dual-Luciferase® Reporter 1000 Assay System(a,b,e)	1,000 assays	E1980

Luciferase Reporter Vectors

Product	Size	Cat.#
pGL3-Control Vector (c,d)	20µg	E1741
pGL3-Enhancer Vector(c,d)	20µg	E1771
pGL3-Promoter Vector(c,d)	20µg	E1761
pGL3-Basic Vector(c,d)	20µg	E1751

Vectors are supplied with a glycerol stock of bacterial strain JM109.

Renilla Luciferase Control Reporter Vectors

Product	Size	Cat.#
pRL-SV40 Vector(f)	20µg	E2231
pRL-TK Vector(f)	20µg	E2241
pRL-CMV Vector(f,g)	20µg	E2261
pRL-null Vector(f)	20µg	E2271

Vectors are supplied with a glycerol stock of bacterial strain JM109. Please call Promega Technical Services or visit our Internet site at www.promega.com to inquire about bulk packaging and pricing information for individual pRL Vectors.

Luminometers (Single Sample)

Product	Cat.#
Turner Designs Luminometer Model TD-20/20 Genetic Reporter Instrumentation Package for Stabilized Assays	E2041
Turner Designs Luminometer Model TD-20/20 Genetic Reporter Instrumentation Package for Stabilized Assays with Printer	E2051
Turner Designs Luminometer Model TD-20/20 Genetic Reporter System with Single Auto Injector	E2351
Turner Designs Luminometer TD-20/20 Genetic Reporter System with Dual Auto Injector	E2361
Turner Designs Luminometer Model TD-20/20 Genetic Reporter Instrumentation Package with Printer, Auto Injector System	E2061

VIII. References

1. Alam, J. and Cook, J.L. (1990) Reporter genes: application to the study of mammalian gene transcription. *Anal. Biochem.* **188**, 245.
2. Wood, K.V. (1991) In: *Bioluminescence and Chemiluminescence: Current Status*, Stanley, P., and Kricka, L., eds., John Wiley and Sons, Chichester, NY, 543.
3. Ow, D.W. *et al.* (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234**, 856.
4. de Wet, J.R. *et al.* (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725.
5. Wood, K.V. (1990) Firefly luciferase: A new tool for molecular biologists. *Promega Notes* **28**, 1.
6. Seliger, H.H. and McElroy, W.D. (1960) Spectral emission and quantum yield of firefly bioluminescence. *Arch. Biochem. Biophys.* **88**, 136.
7. Wood, K.V. *et al.* (1984) Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns. *Biochem. Biophys. Res. Comm.* **124**, 592.
8. de Wet, J.R. *et al.* (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **82**, 7870.
9. Wood, K.V. (1991) In: *Bioluminescence and Chemiluminescence: Current Status*, Stanley, P. and Kricka, L., eds., John Wiley and Sons, Chichester, NY, 11.
10. Manthorpe, M. *et al.* (1993) Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum. Gene Ther.* **4**, 419.
11. Lu, J. and Jiang, C. (1992) Detergents inhibit chloramphenicol acetyl transferase. *BioTechniques* **12**, 643.
12. *Dual-Luciferase® Reporter Assay System Technical Manual* #TM040, Promega Corporation.
13. Carswell, S. *et al.* (1989) Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. *Mol. Cell Biol.* **9**, 4248.
14. Levitt, N. *et al.* (1989) Definition of an efficient synthetic poly(A) site. *Genes Dev.* **3**, 1019.



15. Evans, M.J. and Scarpulla, R.C. (1989) Introns in the 3'-untranslated region can inhibit chimeric CAT and beta-galactosidase gene expression. *Gene* **84**, 135.
16. Kozak, M. (1989) The scanning model for translation: an update. *J. Cell Biol.* **108**, 229.

(a) U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289, European Pat. No. 0 553 234 and Japanese Pat. No. 3171595 have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay. Other patents are pending.

(b) Certain applications of this product may require licenses from others.

(c) The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

(d) U.S. Pat. No. 5,670,356 has been issued to Promega Corporation for a modified luciferase technology.

(e) U.S. Pat. No. 5,744,320 and Australian Pat. No. 721172 have been issued to Promega Corporation for quenching reagents and assays for enzyme-mediated luminescence. Other patents are pending.

(f) Licensed under U.S. Pat. Nos. 5,292,658, 5,418,155 and other patents.

(g) The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

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Luciferase Assay System: *Experienced User's Protocol*

This quick protocol is intended as an easy-to-follow reminder for experienced users. Please follow the complete protocol (Sections III–IV) the first time you use the Luciferase Assay System.

Luciferase Assay Reagent Preparation (Section III.B)	<ol style="list-style-type: none"> 1. Add Luciferase Assay Buffer to the vial containing the Luciferase Assay Substrate. Dispense into aliquots before freezing to avoid repeated freeze-thaw cycles.
Preparation of Mammalian Cell Lysates (Section III.D)	<ol style="list-style-type: none"> 1. Remove growth media from cultured cells. 2. Rinse cells in 1X PBS. Do not dislodge cells. Remove as much of the PBS rinse as possible. 3. Dispense a minimal volume of 1X lysis buffer (CCLR, RLB or PLB) into each culture vessel. For culture dishes, scrape cells, vortex and centrifuge at 12,000 × <i>g</i> to pellet debris. Save supernatant. 4. Proceed to Luciferase Assay using standard conditions.
Preparation of Plant Tissue (Section III.E)	<ol style="list-style-type: none"> 1. Quick-freeze the tissue in liquid nitrogen, grind the frozen tissue to a powder and resuspend in room temperature 1X CCLR with further homogenization. 2. Centrifuge to pellet debris. 3. Proceed to Luciferase Assay using standard conditions.
Preparation of Bacterial Cell Lysate (Section III.E)	<ol style="list-style-type: none"> 1. Mix 40μl nontransformed bacteria (carrier cells) with 50μl of transformed culture. 2. Add 10μl of 1M K₂HPO₄ (pH 7.8) and 20mM EDTA. 3. Quick-freeze on dry ice, and then equilibrate to room temperature by placing the tube in room temperature water. 4. Add 300μl freshly prepared lysis mix (Section VI). Mix and incubate for 10 minutes at room temperature. 5. Proceed to Luciferase Assay using standard conditions.
Protocol for Manual Lumino-meters (Section IV.A)	<ol style="list-style-type: none"> 1. Dispense 100μl of the Luciferase Assay Reagent into the appropriate number of luminometer tubes. 2. Program the luminometer to perform the appropriate delay and measurement times. Typically these are 2 and 10 seconds, respectively. 3. Add 20μl of cell lysate to the luminometer tube and mix by pipetting. 4. Initiate reading. The read times may be reduced if sufficient light is produced. Record the results.
Protocol for Single-Tube Lumino-meters with Injectors (Section IV.B)	<ol style="list-style-type: none"> 1. Prime the luminometer injector with Luciferase Assay Reagent. 2. Dispense 20μl of cell lysate into a luminometer tube. 3. Program the luminometer to perform the appropriate delay and measurement times. Typically these are 2 and 10 seconds, respectively. 4. Place the tube in the luminometer and initiate the reading by injecting 100μl of Luciferase Assay Reagent. Record the results.
Protocol for Plate Reading Lumino-meters (Section IV.C)	<ol style="list-style-type: none"> 1. Program the luminometer to perform the appropriate delay and measurement times. Typically these are 2 and 10 seconds, respectively. 2. Add 20μl of cell to plate wells. Place the plate on the luminometer. 3. Using the injector, add 100μl of Luciferase Assay Reagent to one plate well. 4. Measure and record the light produced. Repeat Steps 3 and 4 for each well.

EXHIBIT B

Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1

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Thrombospondin-1 (TSP-1) is a naturally occurring inhibitor of angiogenesis that limits vessel density in normal tissues and curtails tumor growth. Here, we show that the inhibition of angiogenesis *in vitro* and *in vivo* and the induction of apoptosis by thrombospondin-1 all required the sequential activation of CD36, p59^{lck}, caspase-3 like proteases and p38 mitogen-activated protein kinases. We also detected increased endothelial cell apoptosis *in situ* at the margins of tumors in mice treated with thrombospondin-1. These results indicate that thrombospondin-1, and possibly other broad-spectrum natural inhibitors of angiogenesis, act *in vivo* by inducing receptor-mediated apoptosis in activated microvascular endothelial cells.

Naturally occurring inhibitors of angiogenesis are found in mammalian tissues, where they help maintain the quiescence of the normal vasculature^{1,2}. Many of these compounds are being developed as therapeutic agents to halt the progression of angiogenesis-dependent diseases like cancer. Although such inhibitors can suppress the growth of tumors in animals³⁻⁵ and sometimes drive large tumors into dormancy⁶ by preventing essential neovascularization, their mechanism of action is not yet clear. Several of the broad-spectrum inhibitors, including thrombospondin-1 (TSP-1; ref. 7), angiostatin⁸⁻⁹, endostatin¹⁰ and 2-methoxyestradiol¹¹, can induce apoptosis in cultured cells derived from the vascular endothelium, indicating that they may inhibit angiogenesis by destroying the microvascular endothelial cells that are forming new vessels. The data presented here strongly support this hypothesis for one of these inhibitors, TSP-1.

TSP-1 is a large protein secreted by many cell types that is a potent inhibitor of neovascularization¹²⁻¹⁴. A true natural inhibitor, when it is produced at normal levels in wild-type animals, it limits vessel density¹⁵. When it is delivered in excess systemically, TSP-1 suppresses angiogenic responses in experimental animals³ and thereby slows the growth of tumors^{3,16-18}. Both *in vitro* and *in vivo*, TSP-1 can make endothelial cells unable to respond to a wide variety of inducers of angiogenesis that produce stimulatory signals through very different pathways¹⁹. Therefore, the mechanism by which TSP-1 acts to inhibit angiogenesis must be a broad-based one that functions inside the endothelial cell.

The inhibitory effect of TSP-1 measured

using *in vitro* angiogenesis assays requires the transmembrane receptor CD36 (ref. 20). Here, we demonstrate that CD36 is also essential for the inhibition of neovascularization *in vivo* by TSP-1, and identify the Src family kinase p59^{lck}, caspase 3-like proteases and the stress-activated p38 mitogen-activated protein kinases (MAPKs) as signaling elements that are also required for inhibition of neovascularization by TSP-1. The signaling cascade that mediated the antiangiogenic effect of TSP-1 *in vivo* was also responsible for the induction of apoptosis by TSP-1 in microvascular endothelial cells. In addition, systemic treatment of mice with TSP-1 increased the number of apoptotic endothelial cells in areas of tumor neovascularization. These data indicate that the induction of apoptosis by TSP-1 is an essential component of its ability to inhibit angiogenesis.

CD36 mediates TSP-1 antiangiogenic activity *in vivo*

The first step in TSP-1 inhibition of angiogenesis in *in vitro* assays is its binding to the transmembrane receptor CD36 (ref.

Table 1 Inhibition of neovascularization *in vivo* by TSP-1 requires the presence of CD36 and *lyn*

Additions to implants	Positive corneas / total implanted				
	Wild-type mice		CD36-null mice	<i>lyn</i> -null mice	p53-null mice
	B16	B16/129	(B16/129)	(B16/129)	(B16/129)
bFGF	4/4	2/2	6/6	4/4	6/6
TSP-1	0/3	0/2	0/4	0/4	0/5
bFGF + TSP-1	0/7	0/3	7/7	6/6	1*/7
Angiostatin	0/2	0/2	0/4	0/2	1*/4
bFGF + angiostatin	0/3	1/3	2/7	1*/3	7(1*)/8

Corneal micropocket assays in wild-type and transgenic mice. *, weakly positive response.

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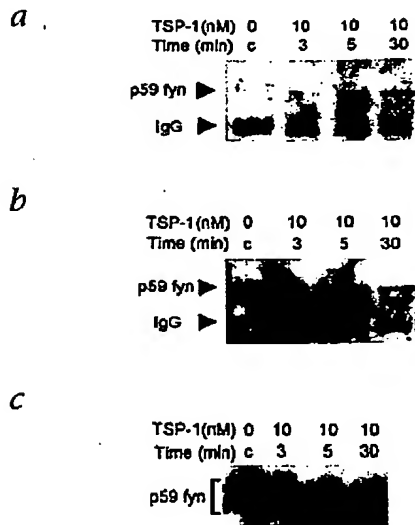


Fig. 1 TSP-1-induced association of activated p59^{fyn} with CD36 in human microvascular endothelial cells. HMVECs were treated with TSP-1 (concentrations and times, above blots), and cell lysates were immunoprecipitated using monoclonal antibody against CD36. **a**, Immunoblot developed with a polyclonal antibody specific for p59^{fyn}. **b**, Immunoblot developed with antibodies against phosphotyrosine. **c**, Autophosphorylation assay of immunoprecipitated complexes. **c**, untreated control.

20). CD36 was also essential for the antiangiogenic activity of TSP-1 *in vivo*. TSP-1 could not block corneal neovascularization induced by basic fibroblast growth factor (bFGF) in mice null for the CD36 receptor (Table 1). These mice were not generically insensitive to inhibitors of angiogenesis, as the function of another inhibitor, angiotensin⁴, was unaffected by the lack of CD36 (Table 1). Angiotensin and TSP-1 also differed in their dependence on p53, as only TSP-1 could inhibit neovascularization effectively in the corneas of p53-null mice (Table 1). As expected of a TSP-1 receptor, CD36 may be important in the natural regulation of organ vessel density. In the brain, an organ with high levels of TSP-1 that is initially vascularized by angiogenesis, vessel density was significantly increased in mice null for CD36 (68 ± 0.40 vessels per high-powered field, compared with only 41 ± 0.38 vessels per high-powered field for wild-type mice; $P < 0.01$).

CD36 signals through p59^{fyn}

CD36 can associate Src-family tyrosine kinases²¹, and in microvascular endothelial cells it binds most strongly to p59^{fyn} (ref. 22), although this association has not been linked to any biological response. Therefore, we used mice null for *fyn* to determine if p59^{fyn} was an essential component of the signaling cascade that results in inhibition of neovascularization by TSP-1. bFGF stimulated corneal neovascularization equally well in *fyn*-null and wild-type mice, but in the *fyn*-null mice, TSP-1 was unable to block angiogenesis (Table 1). Angiotensin was effective in the absence of p59^{fyn}, showing that the null mice were sensitive to other antiangiogenic agents, even those that cause endothelial cell apoptosis⁸⁻⁹. We sought to determine if p60^{src}

was also relevant to the antiangiogenic effect of TSP-1, but p60^{src} null mice failed to respond to common inducers of neovascularization, precluding these studies.

As new blood vessels that form during *in vivo* angiogenesis arise exclusively from the microvasculature, we used cultured human dermal microvascular endothelial cells (HMVECs) to identify the endogenous signaling elements that mediate the antiangiogenic effect of TSP-1. Treatment of HMVECs with TSP-1 at a concentration known to inhibit endothelial cell chemotaxis²³ resulted in increased recruitment of p59^{fyn} to complexes containing CD36 (Fig. 1a), increased tyrosine phosphorylation of CD36-associated p59^{fyn} (Fig. 1b), and increased incorporation of ³²P into a 59-kDa phosphoprotein in CD36 immunocomplexes with kinetics that paralleled those for the receptor/kinase association and the tyrosine phosphorylation of p59^{fyn} (Fig. 1c).

Caspase-dependent activation of p38MAPK

The stress-responsive kinases JNK and p38MAPK are often activated by apoptotic signaling cascades^{24,25} and can be essential in the induction of apoptosis in some cells^{26,27}. In microvascular endothelial cells, p38MAPK was activated by TSP-1 in a time- and dose-dependent manner (Fig. 2a). Activation was mediated by CD36, as antibodies that blocked TSP-1 access to CD36 (FA6-152; ref. 20) prevented p38MAPK activation by TSP-1 but did not influence activation of p38MAPK by osmotic shock (Fig. 2b and c). We obtained similar results whether we measured p38MAPK activation by staining for the phosphorylated active form (Fig. 2a and b) or by the ability of p38MAPK to phosphorylate its substrate, ATF-2 (Fig. 2c).

Activation of p38MAPK required p59^{fyn}. As CD36-expressing human microvascular cells are not easily transfected, we used microinjection of antibodies to inactivate intracellular signaling molecules. Neutralizing antibodies against Fyn injected into microvascular endothelial cells blocked the activation and nuclear localization of p38MAPK, as detected by the antibody specific for its active, dually phosphorylated form, whereas isotype-matched control antibodies had no such effect (Fig. 2e).

Seeking a link between Fyn and p38MAPK, we found that the activation of p38MAPK in endothelial cells in response to TSP-1 was blocked by caspase inhibitors. zVAD-fmk, a broad-spectrum inhibitor of caspases, prevented TSP-1 activation of p38MAPK (data not shown). In addition, the more specific caspase inhibitor DEVD-CHO, which blocks caspase 3-like proteases, prevented the activation of p38MAPK (Fig. 2d), whereas YVAD-CHO, an inhibitor of ICE/caspase 1-like proteases, did not (Fig. 2d).

We could also detect the induction of caspase 3-like activity by TSP-1 in endothelial cells directly by measuring proteolytic activity in endothelial cell lysates using a caspase 3-specific chromogenic substrate (Fig. 3). Two peaks of activity were separated by a temporary decrease (Fig. 3). The small but important increase in caspase 3-like activity 15 minutes after the addition of TSP-1 was not curtailed by blocking p38MAPK with SB203580, a specific p38MAPK inhibitor²⁸. The later increase in caspase 3-like activity was eliminated when p38MAPK was blocked by SB203580 (Fig. 3).

The activation of p38MAPK was essential to the inhibition of angiogenesis by TSP-1 both *in vitro* and *in vivo*. We assessed the migration of bovine capillary endothelial cells up a gradient of bFGF gradient in a Boyden chamber, as an *in vitro* assay²⁹. These bovine cells are inhibited by TSP-1 in a CD36-dependent man-

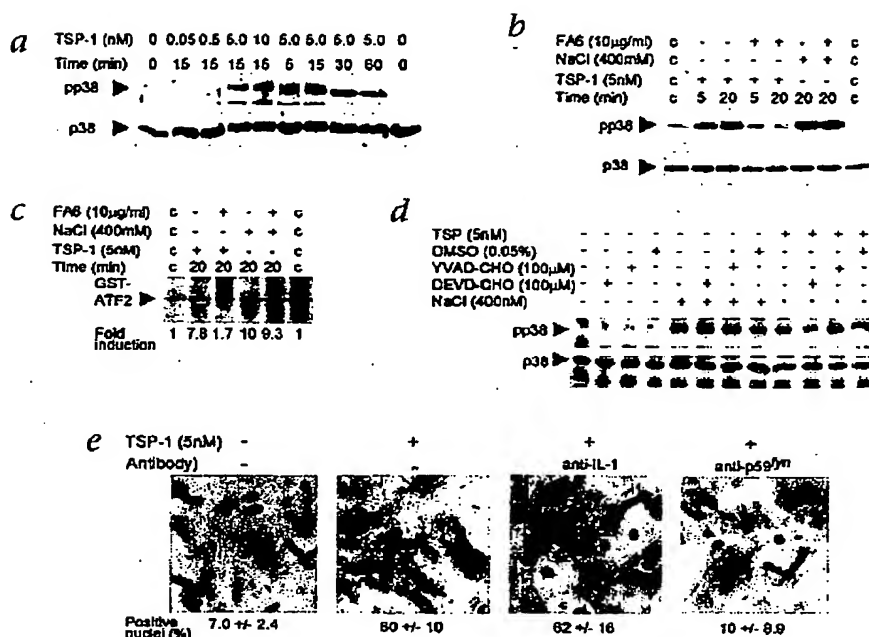


Fig. 2 p38 MAP kinase activation by TSP-1 in microvascular endothelial cells and requirement for CD36 engagement, Fyn and caspases. **a–c**, HMVECs were treated with TSP-1 alone or in combination with antibodies that block access to CD36, or with NaCl (positive control), and were assayed for activation of p38 MAP kinase by developing western blots of cell lysates with antibodies against active p38 (pp38) or against total p38 (p38) as a loading control (**a** and **b**) or by immunocomplex kinase assay (**c**). Treatment conditions, above blots. c, untreated control. Numbers below figure (**c**), relative quantification by phosphorimager. **d**, HMVECs were

treated for 20 min with TSP-1 alone or in combination with 100 µM DEVD-CHO (caspase 3 inhibitor) or YVAD-CHO (ICE/caspase 1 inhibitor) or with DMSO solvent and assayed by developing western blots of cell lysates with antibodies against active p38 (pp38) or against total p38 (p38) as a loading control. **e**, HMVECs were microinjected with antibodies against Fyn (anti-p59[™]) or with control antibodies (anti-IL-1) and subsequently treated with TSP-1 for 30 min, fixed, and stained to detect the nuclear localization of activated p38 MAP kinase. Below, % cells showing nuclear localization of activated p38MAPK ± s.e.m.

ner²⁰ and are routinely used in migration assays, as their response is very reproducible. TSP-1 could no longer inhibit the directed migration of endothelial cells in this assay when the p38MAPK inhibitor SB203580 was present (Fig. 4a). Similarly, SB203580 prevented TSP-1 inhibition of bFGF-induced neovascularization in the mouse cornea (Table 2). SB203580 had no effect when tested alone on bFGF-induced angiogenesis either *in vitro* or *in vivo*.

TSP-1 induces apoptosis

The biological result of TSP-1 activation of the CD36–p59[™]–caspase 3–p38MAPK cascade in microvascular endothelial cells

was apoptosis. Endothelial cells treated *in vitro* with TSP-1 became apoptotic, as shown by TUNEL assay (Fig. 4b–d). When we measured apoptosis independently by nuclear morphology of propidium iodide-stained cells, the number of apoptotic cells increased from 2.1 ± 1.0 % in untreated cultures to 17.6 ± 4.2 % after TSP-1 treatment. The apoptotic response was sensitive to antibodies that neutralized TSP-1 (A4.1) or that blocked access of TSP-1 to CD36 (FA6; Fig. 4b). Treatment of cells with an IgM monoclonal antibody that activates CD36 (SMΦ; ref. 20) induced apoptosis, verifying the role of CD36 in this response (Fig. 4b). TSP-1 was unable to induce apoptosis in the presence of compounds that inhibited p38MAPK (Fig. 4b) or caspase 3-like proteases (Fig. 4d), but its effect was not hampered by two compounds that blocked caspase 1-like proteases. TSP-1-induced apoptosis was also not reduced when the extracellular signal-regulated kinase/ERK cascade was blocked with PD98059 (Fig. 4b). This finding is consistent with our observation that TSP-1 did not interfere with the activation of p42 ERK and p44 ERK-1 by bFGF (data not shown) and thus is not blocking the early incoming signals

Table 2 Inhibition of neovascularization *in vivo* by TSP-1 requires the activation of p38MAPK and caspases

Additions to implants	Inhibitor added:	Positive corneas/total implanted			
		none	z-VAD-fmk (vs. caspases)	DEVD-CHO (vs. caspase 3-like proteases)	SB203580 (vs. p38MAPK)
none		0/4	0/4	0/2	0/4
bFGF		10/10	4/4	2/2	4/4
TSP-1		0/6	1/4	ND	1*/4
bFGF + TSP-1		1*/11	4/4	4/4	6/6

Corneal micropocket assay in wild-type mice. vs, against; *, weakly positive response.

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from the bFGF receptor on the endothelial cells.

Although our TSP-1 preparation contained no detectable transforming growth factor (TGF)- β , it is always possible that during the relatively long apoptosis assay, TSP-1 might activate the latent TGF- β that can be produced by the target endothelial cells³⁰, and that this in turn might induce apoptosis. However, this did not occur. Neutralizing antibodies effective against TGF- β s did not diminish TSP-1-induced apoptosis (Fig. 4c).

TSP-1 seemed to activate the same set of signaling molecules to induce apoptosis as it did to inhibit angiogenesis, indicating that the induction of apoptosis might be essential for the inhibition of angiogenesis by TSP-1. Such an interpretation is supported by the finding that the ability of TSP-1 to inhibit endothelial cell migration (Fig. 4a), like its ability to induce apoptosis (Fig. 4a, b and d) was blocked by caspase inhibitors. Moreover, the median effective dose (ED₅₀) for TSP-1 induction of apoptosis derived from dose response curves was 8 ± 4 nM, a value very similar to the ED₅₀ for TSP-1 inhibition of migration³⁴. As for TSP-1 inhibition of new vessel formation *in vivo*, the caspase inhibitors that were effective *in vitro* in blocking endothelial cell migration (z-VAD; Fig. 4a) and apoptosis (z-VAD and DEVD; Fig. 4d) also prevented TSP-1 inhibition of corneal neovascularization *in vivo* (Table 2).

Seeking additional evidence that TSP-1 acts *in vivo* to inhibit angiogenesis by inducing apoptosis, we sought to identify apoptotic endothelial cells *in vivo* after TSP-1 treatment of

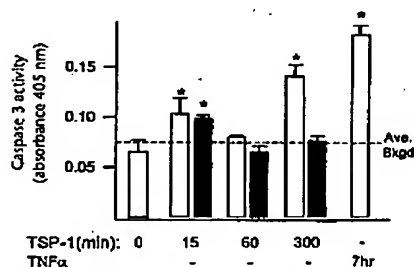


Fig. 3 p38MAPK-dependent activation of caspase 3-like activity. HMVECs were treated with TNF α or with TSP-1 (times, below graph) in the presence (■) or absence (□) of the p38 inhibitor SB203580, and cell lysates were assayed for caspase 3-like activity. *, $P < 0.05$, compared with average background (Ave. Bkgd).

tumor-bearing mice. Melanoma cells seeded in the lungs of nude mice seldom develop into tumors large enough to see with the naked eye if mice have been treated systemically with injections of TSP-1 (ref. 3). We compared tissue sections from TSP-1-treated mice containing small tumors with similar sections derived from mice treated with saline (vehicle) only; there was a considerable increase in the percentage of apoptotic endothelial cells in the treated group, mainly in the area of active neovascularization surrounding the tumor (Fig. 5). When we identified apoptotic cells by annexin V staining, and endothelial cells by CD34, apoptotic endothelial cells increased from $6 \pm 1.5\%$ to $15 \pm 1.8\%$. When we used TUNEL as an apoptosis marker and CD31 for endothelial cells (Fig. 5), the increase was from $12 \pm 2.1\%$ to $28 \pm 2.7\%$ (Fig. 5g). There was some apoptosis in endothelial cells from vehicle-treated mice (Fig. 5a-c), as has been seen in remodeling tissue³¹. In both treated and control mice, the capillaries lining the alveolar sacs of normal lung tissue were mostly TUNEL-negative.

Discussion

TSP-1 is one of the first of the natural inhibitors of angiogenesis to have an *in vivo* mechanism of action defined. Evidence is

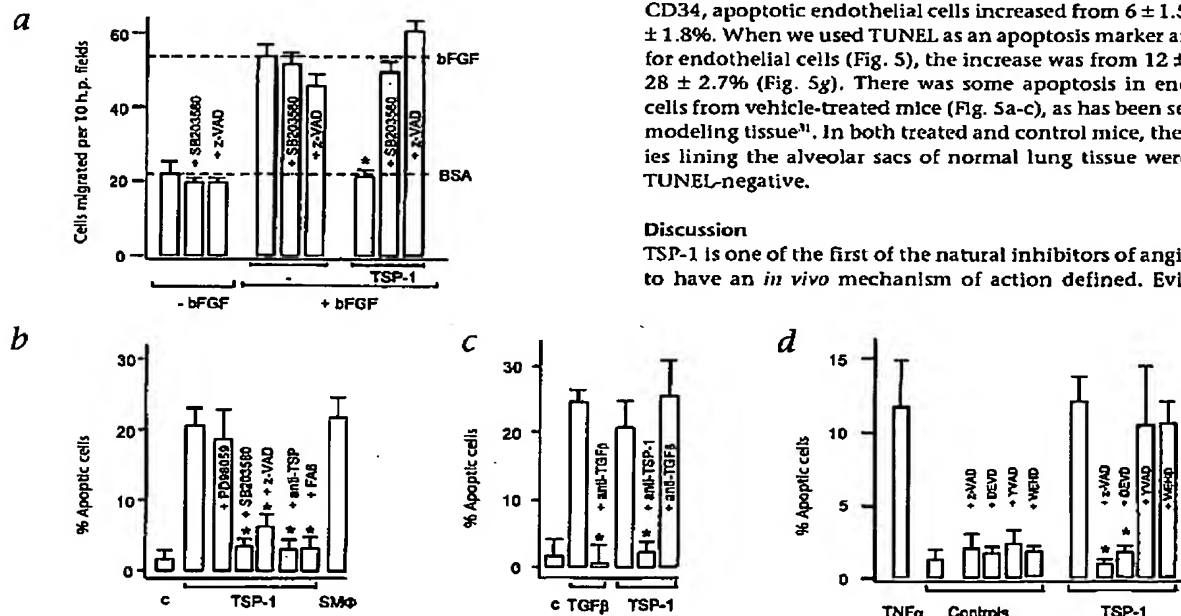


Fig. 4 Prevention of TSP-1 repression of endothelial cell migration and TSP-1 induction of apoptosis by inhibition of p38MAPK and caspases. **a**, BCECs were allowed to migrate towards bFGF in the presence or absence of TSP-1 alone or in combination with SB203580 (p38MAPK inhibitor) or z-VAD-fmk (caspase inhibitor). Vertical axis, cells migrated per 10 high-power fields. Right margin: BSA, background migration in the absence of a chemotactic gradient; bFGF, migration towards bFGF. Data include s.e.m. *, $P < 0.002$ compared with bFGF alone. **b** and **c**, The ability of TSP-1 (b) and TGF- β 1 (c) to induce apoptosis in HMVECs (b) or in BCECs (c) in the presence of 0.5% serum was assessed by

TUNEL staining in the presence and absence of antibodies that neutralize TSP-1 (anti-TSP-1) or TGF- β s (anti-TGF- β) or block access to CD36 (FA6) and in the presence of a specific inhibitor of MEK (PD98059) or of p38 MAPK (SB203580) or of caspases (z-VAD). **c**, serum alone, at a concentration of 0.5% (negative control); SM Φ , CD36 agonist. Data include s.e.m. *, $P < 0.008$, compared with TSP-1 and TGF- β , respectively. **d**, The effect of inhibitors of multiple caspases (z-VAD), of caspase 3-like activity (DEVD) and of ICE/Caspase 1-like activity (YVAD and WEHD) on TSP-1-induced apoptosis was measured by TUNEL staining. Data include s.e.m. *, $P < 0.001$, compared with TNF- α .

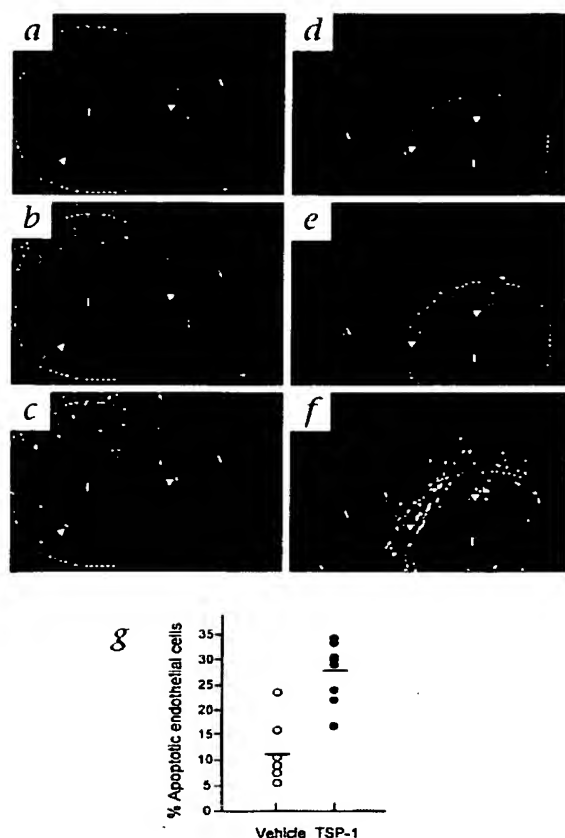


Fig. 5 *In situ* identification of apoptotic endothelial cells. Mice whose lungs had been seeded with B16F10 melanoma tumor cells were treated with thrombospondin-1 or saline (vehicle), and their lungs were fixed and stained for endothelial cells (CD31; red) and for apoptotic cells (TUNEL; green). Dotted lines outline tumor tissue (T). A, alveolar spaces; arrows, apoptotic endothelial cells. **a–c**, Tissue from 'sham-treated' mouse stained for endothelial cells (**a**) or apoptotic cells (**b**). **c**, Merge of images **a** and **b**. **d–f**, Tissue from TSP-1-treated mice stained for endothelial cells (**d**) or apoptotic cells (**e**). **f**, Merge of images **d** and **e**. Scale bars represent 20 μ m. **g**, Quantification of apoptotic endothelial cells adjacent to tumors. Averages in the two groups (solid lines) were significantly different ($P < 0.003$).

strong that TSP-1 works to inhibit angiogenesis *in vivo* by inducing apoptosis of endothelial cells that are forming new vessels. As shown here, the activation of each of the four different molecules, CD36, pp59^{src}, caspases and p38MAPK, was essential for the induction of apoptosis by TSP-1 in microvascular cells growing *in vitro*. Although none were required for the induction of new vessels in the cornea by bFGF, each was essential for the inhibition of this angiogenesis by TSP-1. These correlations combined with the direct demonstration that TSP-1 inhibition of corneal neovascularization was sensitive to DEVD-CHO, an inhibitor specific for the group II caspases that are uniquely associated with apoptosis, indicate that the induction of apoptosis is essential for TSP-1 inhibition of angiogenesis. In addition, an increase in apoptotic endothelial cells could be detected *in situ* in vessels developing around tumors in TSP-

1-treated mice.

Our observation that the ability of TSP-1 to induce apoptosis in cultured endothelial cells accurately predicted a requirement for apoptosis *in vivo* indicates that other inhibitors of angiogenesis, such as angiostatin, that also induce apoptosis *in vitro* may act like TSP-1 *in vivo*. Although the two compounds differed in that only angiostatin required p53, their lack of synergy *in vitro* (O.V.V. and N.B., unpublished data) makes it likely that their distinct pathways converge at some point in the endothelial cell.

Whether or not a particular vessel is stimulated to sprout *in vivo* is thought to depend on the balance of positive and negative signals in its microenvironment^{1,2}. It is possible that one way the endothelial cell weighs the effects of these opposing signals is by using its apoptotic apparatus. Other cells are known to live or die depending on their relative exposures to signals that activate or suppress apoptosis⁴², integrating opposing signals at the mitochondrial outer membrane and possibly elsewhere in the cell⁴³. Endothelial cells, particularly those involved in an angiogenic response, are in a similar situation. Inducers of angiogenesis can serve as survival factors for endothelial cells^{44–46}, stimulating the production of anti-apoptotic molecules and inhibiting the sustained activation of stress-activated kinases. Many of the inhibitors of angiogenesis are pro-apoptotic, and some, like 2-methoxyestradiol⁴⁷, induce apoptosis-associated stress-activated kinases that we have shown here to be essential for TSP-1 inhibition. These facts, when considered with our demonstration that apoptosis can be an essential factor in the inhibition of angiogenesis *in vivo*, raise the possibility that one way that endothelial cells 'decide' whether or not to initiate and/or to sustain an angiogenic response is by integrating pro- and anti-apoptotic signals, forming new vessels only if survival signals are dominant. Thus, the regulation of the apoptotic machinery serves as a 'fulcrum' on which the cells 'balance' the effects of stimulators and inhibitors of neovascularization.

We have put in order the individual proteins identified in this work as essential for TSP-1 inhibition of angiogenesis and have thus formed a skeletal outline of a signaling cascade (Fig. 6). It is apparent that CD36 interacts with pp59^{src}, but links from pp59^{src} to caspase activation are unknown. The Src kinases are often redundant, substituting for one another in many complex biological processes⁴⁸, thus our specific *in vivo* requirement for Fyn is unusual. However, there are two other physiological processes for which Fyn activity is indispensable: neural responses to ethanol and some aspects of long-term learning in mice^{49,50}. The specificity of these neural functions for Fyn may depend on a specific isomer found in the brain. It is not known if unusual forms of Fyn also occur in endothelial cells, nor is it yet apparent if TSP-1 inhibition of angiogenesis requires both Fyn and another of the Src kinases such that removal of either one of them would abrogate function.

The ubiquitously expressed stress-activated protein kinases JNK and p38MAPK are often activated by apoptotic signaling cascades⁵¹, and our data indicate that a caspase 3-like protease is required for the activation of p38MAPK. Activation of stress-activated kinases by a caspase-dependent mechanism has been seen before in cells progressing to apoptosis^{52,53}, as caspase cleavage can directly activate the upstream kinase MEKK-1 (ref. 41). Presumably these kinases in turn activate nuclear transcription, and TSP-1-induced apoptosis did seem to require new protein synthesis, as it was sensitive to cycloheximide (data not shown), but the identity of essential new proteins and how they are

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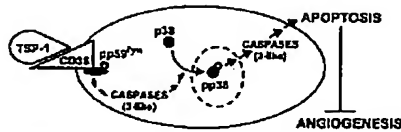


Fig. 6 Signaling molecules induced by TSP-1 in microvascular endothelial cells essential for inhibition of angiogenesis. All elements are essential for the inhibition of endothelial cell migration *in vitro*, for apoptosis *in vitro* and for the inhibition of neovascularization *in vivo*. The sequence in which they act is correct, although physical associations have been demonstrated only for TSP-1 and CD36 and for CD36 and p38⁹. Multiple arrows, undefined intermediates.

linked to the apoptotic apparatus remain to be determined.

TSP-1 is very effective *in vitro* in inhibiting the migration of microvascular endothelial cells up a gradient of an angiogenic inducer, an activity that depends in large part on chemotaxis and is evident in a 4-hour assay. The ability of TSP-1 to block this migration was sensitive to two inhibitors that also blocked apoptosis, SB203580 and z-VAD-fmk. As caspase 3-like activity could be detected as early as 15 minutes into TSP-1 treatment and an increase in TUNEL-positive cells could be seen by 2–4 hours (data not shown), it is possible that endothelial cells committing to apoptosis may become unable to respond to chemotactic stimuli. Conversely, the inhibition of chemotaxis may be an early harbinger of apoptosis in endothelial cells. The ability of caspases to cleave gelsolin⁴² provides one possible explanation for the link between apoptosis and chemotaxis, as gelsolin is increased in migrating endothelial cells⁴³ and is cleaved early in the apoptotic program⁴².

Available data indicate that, like the integrin antagonists that interfere with the survival of only activated endothelial cells⁴⁴, TSP-1 induces apoptosis mainly in endothelial cells that are activated or stressed⁷. Endothelial cells cultured *in vitro* were sensitive to TSP-1 when starved or in low serum; their sensitivity doubled when they were exposed to activating concentrations of bFGF (data not shown). *In vivo*, TSP-1 did not damage the existing limbal vessels during the cornea assay, and mice exposed systemically to antiangiogenic levels of TSP-1 for several weeks showed no visible abnormalities⁴. In addition, the endothelial cell apoptosis associated with tumors in TSP-1 treated mice was most prominent at the periphery of the tumor, where new vessels are actively developing. Although TSP-1 can bind $\alpha_v\beta_3$ integrins, and interference with these integrins can drive activated endothelial cells into apoptosis⁴⁴, TSP-1 did not seem to inhibit angiogenesis through an integrin-dependent mechanism. Fragments of TSP-1 lacking integrin binding motifs were also able to induce apoptosis (data not shown) and to inhibit angiogenesis²⁴ and, unlike the integrin antagonists⁴⁴, TSP-1 was effective in p53-null mice (Table 1).

The data presented here indicate that TSP-1 inhibits angiogenesis by inducing apoptosis in activated endothelial cells, a mechanism that explains its ability to prevent the cells from responding to a wide variety of pro-angiogenic factors that signal through different pathways¹⁹. They also identify several members of a cascade of sequential signaling molecules whose activation is essential to this apoptosis (Fig. 6) and that themselves could be targets of future intervention strategies designed to prevent endothelial cells from forming the new vessels on which tumors so heavily depend.

Methods

Cells and proteins. Human dermal microvascular endothelial cells (HMVECs) from Cell Systems (Kirkland, Washington) or from Cascade Biologics (Bethlehem, Pennsylvania) were maintained in endothelial cell growth media (Clonetics, San Diego, California) and used at passage numbers 4–8. Bovine adrenal capillary endothelial cells BP10T8 (BCECs) provided by J. Folkman (Children's Hospital, Harvard Medical School, Boston), were maintained in DME with 10% donor calf serum (IHR Biosciences, Lenexa, Kansas), 100 μ g/ml endothelial cell mitogen (Biomedical Technologies, Stoughton, Massachusetts), 2 mM glutamine, and were used at passage numbers 13–14. Human thrombospondin-1 was purified from human platelet releasate¹ and was stored at -70°C in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 0.1 mM CaCl_2 . It was used at a more than 100-fold dilution in cellular assays, a more than 10-fold dilution for corneal assays, and was neutralized with A4.1 (ref. 3).

Angiogenesis assays. *In vitro* endothelial cell migrations were done in quadruplicate as described²⁹ in a modified Boyden chamber using DME with 0.1% BSA as a negative control and bFGF at 10 ng/ml as a positive control. BCECs were treated with 10 μ M SB203580 (CalBiochem, San Diego, California), that at this concentration is specific for p38MAPK, or with 100 μ M z-VAD-fmk (Enzyme Systems, Livermore, California), a caspase inhibitor. These inhibitors were present during the 2 h the cells were adhering to the membrane and during the 3- to 4-hour migration.

For *in vivo* angiogenesis assays, Hydron/Sucrafate pellets were formulated and implanted into the avascular corneas of wild-type mice or various strains of transgenic mice as described³. The vigorous ingrowth of new vessels after 5 d was considered a positive response. Wild-type, *lyn*-null mice and p53-null mice were from Jackson Laboratories (Bar Harbor, Maine); CD36-null mice have been described⁴⁵. All compounds in the pellets were used at concentrations at least 10-fold higher than those used in the migration assay, to account for the diffusion from a slow-release pellet. Where indicated, the pellets contained 50 ng bFGF, 200 ng TSP-1, 200 ng angiostatin, 100 μ M SB203580, 1 mM z-VAD-fmk or DEVD-CHO (Biolmol, Plymouth Meeting, Pennsylvania). Like other inhibitors of angiogenesis, the human TSP-1 is routinely used in the mouse or rat cornea despite species differences, as the cornea is an immune-privileged site⁴⁶. Because null mouse mutations were on a Bl6/129 background, both Bl6 mice and the F2 generation of Bl6/129 hybrid mice (Jackson Laboratories, Bar Harbor, Maine) were used as controls.

Apoptosis assays. To assay apoptosis *in vitro*, endothelial cells were grown to confluence on 8-mm gelatinized coverslips and were incubated overnight with 10 nM TSP-1 alone or in the presence of 10 μ M PD98059 (an inhibitor of the MEK kinase) (CalBiochem, La Jolla, California), 10 μ M SB203580 or 100 μ M z-VAD-fmk, DEVD-CHO, YVAD-CHO (Biolmol, Plymouth Meeting, Pennsylvania) or WEHD-fmk (Enzyme Systems, Livermore, California), 10 μ g/ml antibody against TSP-1 (A4.1, ref. 3) or 2 μ g/ml of the blocking monoclonal IgG antibody FA6-152 (Immunotech, Miami, Florida). The monoclonal IgM antibody SM Φ (Sigma), an agonist for CD36 (ref. 20), was used at a concentration of 1 μ g/ml; TGF- β 1, at a concentration of 2 ng/ml; and neutralizing antibodies, at a concentration of 4 μ g/ml (R & D Systems, Minneapolis, Minnesota). Cells were fixed with 1% paraformaldehyde in phosphate-buffered saline, and apoptotic cells were detected by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling) staining using ApopTagTM Plus kit (Oncor, Gaithersburg, Maryland) with propidium iodide as counterstain. A minimum of 350 cells per condition were visually assessed. Apoptosis was also assessed using nuclear morphology; at least 700 cells were evaluated for each condition.

To measure caspase 3-like activity, HMVECs (Cascade Biologics, Bethlehem, Pennsylvania) were plated at equal density in 60-mm gelatinized tissue culture plates, grown to 80–90% confluence, and treated with 5 nM TSP-1, or with 30 ng/ml TNF α with cycloheximide (2 μ g/ml). After being treated, cells were collected, combined with cells in the culture supernatant and counted. Caspase 3-like enzymatic activity was detected using Apo-Alert Caspase-3 colorimetric assay kit (Clontech, Palo Alto, California). Each cell lysate was also tested in the presence of DEVD-fmk to eliminate caspase 3-like activity. These measurements were combined to yield average background and were compared with experimental points

using a two-tailed t-test.

Apoptosis of endothelial cells *in vivo* was assessed in formalin-fixed tissue from the lungs of nude mice with B16/F10 melanoma tumors as described¹. B16/F10 cells were seeded in the lungs of nude mice through the tail vein, and 24 h later, mice were treated daily with intraperitoneal injections of vehicle phosphate-buffered saline or 10 mg/kg human TSP-1 for 21 d before lungs were collected and the sections were obtained. Sections were deparaffinized, rehydrated, blocked with 1% normal serum for 20 min, and incubated 60 min in 1% blocking solution with goat antibody against mouse CD31 (PECAM-1, Santa Cruz Biotechnology, Santa Cruz, California). After three 5-minute washes with phosphate-buffered saline, secondary antibodies were applied for 60 min at room temperature using a 1:20 dilution of sheep antibody against mouse IgG conjugated with R-phycerythrin (Sigma). An ApopTag kit (Intergen, Purchase, New York) was used according to the manufacturer's instructions. Similar studies were done using mouse monoclonal antibody against human CD34 and rabbit polyclonal antibody against human annexin-V conjugated with fluorescein isothiocyanate (both from Santa Cruz Biotechnology, Santa Cruz, California). Slides were rinsed briefly in phosphate-buffered saline, mounted with Prolong Antifade (Molecular Probes, Eugene, Oregon) and viewed with an Olympus Fluoview Confocal Laser Scanning Microscope. Slides were assigned a code and apoptotic vascular endothelial cells per 250 endothelial cells were counted by experimenters 'blinded' to sample identity. Analysis of dual-stained tissues demonstrated that CD31-stained vessels containing red blood cells were more consistently seen around the edges of the tumor, whereas CD34 occasionally stained circulating hematopoietic cells. TUNEL and annexin V staining were equally effective in detecting apoptotic tumor and endothelial cells.

Statistics for these and other experiments were analyzed on raw data using Student's two-tailed t-test. Data include standard errors, converted to percentiles where necessary.

Biochemical assays. For immunoprecipitation with the monoclonal antibody against CD36 FA6-152 (Immunotech, Westbrook, Maine), HMVECs were grown to confluency, lysed in a modified RIPA buffer, and immunoprecipitated⁴⁰, then immunoblotted with antibodies against Fyn (Upstate Biotechnology, Lake Placid, New York) or against phosphotyrosine (RC20; Transduction Laboratories, Lexington, Kentucky). For Fyn autophosphorylation assays, immunoprecipitates were resuspended in kinase buffer (50 mM HEPES, pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂, 12.5 mM β -glycerophosphate, 0.1 mM sodium orthovanadate and 1 μ M cold ATP/5 μ M γ -³²P-ATP) and incubated 10 min at 30 °C before being separated by standard 8% SDS-PAGE.

For p38 MAPK immunocomplex kinase assays, cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris HCl, pH 7.5, and 2 mM EDTA) supplemented with protease and phosphatase inhibitors. Lysates were cleared by centrifugation and incubated overnight at 4 °C with antibody against p38MAPK (Santa Cruz Biotechnology, Santa Cruz, California) followed by a 1-hour incubation at 4 °C with protein G beads. The immunoprecipitates were washed four times with lysis buffer and once with kinase reaction buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 12.5 mM β -glycerophosphate, 0.1 mM sodium orthovanadate). The reactions with the glutathione S-transferase-activating transcription factor 2 fusion protein (GST-ATF2; 1 μ g/reaction) as a substrate were incubated at 30 °C for 30 min and terminated by addition of 5x electrophoresis sample buffer. Blots were developed with phospho-specific antibodies against p38MAPK (New England Biolabs, Beverly, Massachusetts). After being 'stripped', the membranes were re-probed with antibody against p38MAPK (Santa Cruz Biotechnology, Santa Cruz, California), as a loading control.

All biochemical experiments were repeated three to seven times with similar results. Because of the variations in CD36 expression among batches of HMVECs, results from different experiments were not combined. However, similar results were obtained using at least two independently initiated cultures of HMVECs that at various passages contained between 40 and 70% CD36⁺ cells.

Microinjections. For microinjection cells were plated on gelatinized coverslips with marked microgrids (Celloclate 175; Eppendorf, Westbury, New York) in 24-well multi-well plates at a concentration of 5×10^4 cells/well,

grown overnight, transferred into 35-mm Petri dishes with HEPES-buffered culture media and injected with either monoclonal antibody against Fyn (Fyn15, from Santa Cruz Biotechnology, Santa Cruz, California) (ref. 22) or isotype-matched mouse monoclonal antibody (anti-IL-1; Sigma) using a Zeiss setup of Micromanipulator 5171 and Transjector 5246. All cells within a single grid square were injected similarly. After 16–18 h of recovery, cells were re-fed with serum-free media containing 0.1% BSA and treated, as was the uninjected control, for 20 min with 5 nM human TSP-1, fixed with 2% paraformaldehyde, and stained for phosphorylation/nuclear translocation of p38 MAPK using PhosphoPlus p38 MAP kinase antibody kit (New England Biolabs) as recommended by manufacturer. The staining was visualized using AEC chromogen substrate (BioGenex, San Ramon, California). An average of 178 cells were assessed per condition. Because of variations in CD36 expression among batches of HMVECs, a single batch was used for each experiment, and data from different experiments were not combined. Three experiments were done with similar results, all using HMVECs with 60–70% CD36⁺ cells.

Vessel counts. For vessel counts, representative coronal sections were obtained from the brains of three mice of each genotype, at the mid-parietal region; sections did not include the cerebellum. After sections were stained with hematoxylin and eosin, vessels were counted in ten non-overlapping fields at $\times 40$ magnification.

Acknowledgments

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1. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364 (1996).
2. Bouck, N., Stellmach, V. & Hsu, S. How tumors become angiogenic. *Adv. Cancer Res.* 69, 135–174 (1996).
3. Volpert, O.V., Lawler, J. & Bouck, N.P. A human fibrosarcoma inhibits systemic angiogenesis and the growth of experimental metastases via thrombospondin-1. *Proc. Natl. Acad. Sci. USA* 95, 6343–6348 (1998).
4. O'Reilly, M.S. et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79, 315–328 (1994).
5. O'Reilly, M.S. et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88, 277–285 (1997).
6. Boehm, T., Folkman, J., Browder, T. & O'Reilly, M.S. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* 390, 404–407 (1997).
7. Guo, N., Krutzsch, H.C., Inman, J.K. & Roberts, D.D. Thrombospondin 1 and type I repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. *Cancer Res.* 57, 1735–1742 (1997).
8. Claesson-Welsh, L. et al. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. *Proc. Natl. Acad. Sci. USA* 95, 5579–5583 (1998).
9. Lucas, R. et al. Multiple forms of angiostatin induce apoptosis in endothelial cells. *Blood* 92, 4730–4741 (1998).
10. Dhanabal, M. et al. Endostatin induces endothelial cell apoptosis. *J. Biol. Chem.* 274, 11721–11726 (1999).
11. Yue, T.L. et al. 2-Methoxyestradiol, an endogenous estrogen metabolite, induces apoptosis in endothelial cells and inhibits angiogenesis: possible role for stress-activated protein kinase signaling pathway and Fas expression. *Mol. Pharmacol.* 51, 951–962 (1992).
12. DiPietro, L.A. in *Regulation of Angiogenesis* (eds. Goldberg, I.D. & Rosen, E.M.) 295–313 (Birkhäuser Verlag, Basel, Switzerland, 1997).
13. Roberts, D.D. Regulation of tumor growth and metastasis by thrombospondin-1. *FASEB J.* 10, 1183–1191 (1996).
14. Dawson, D.W. & Bouck, N. in *Antiangiogenic Agents in Cancer* (ed. Teltcher, B.A.) 185–204 (Humana, Totowa, New Jersey, 1998).
15. Crawford, S.E. et al. Thrombospondin-1 is a major activator of TGF- β 1 *in vivo*. *Cell* 93, 1159–1170 (1998).
16. Weinstein-Saslow, D.L. et al. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res.* 54, 6504–6511 (1994).
17. Castle, V.P., Dixit, V.M. & Polverini, P.J. Thrombospondin-1 suppresses tumorigenesis and angiogenesis in serum- and anchorage-independent NIH 3T3 cells. *Lab. Invest.* 77, 51–61 (1997).
18. Bleuel, K., Popp, S., Fussenig, N.E., Stanbridge, E.J. & Boukamp, P. Tumor suppress-

specific

ARTICLES

- sion in human skin carcinoma cells by chromosome 15 transfer or thrombospondin-1 overexpression through halted tumor vascularization. *Proc. Natl. Acad. Sci. USA* 96, 2065-2070 (1999).
19. Volpert, O.V. et al. Inhibition of angiogenesis by thrombospondin-2. *Biochem. Biophys. Res. Comm.* 217, 326-332 (1995).
 20. Dawson, D.W. et al. CD36 mediates the *in vitro* inhibitory effects of thrombospondin-1 on endothelial cells. *J. Cell Biol.* 138, 707-717 (1997).
 21. Huang, M.M., Bolen, J.B., Bantwell, J.W., Shatill, S.J. & Brugge, J.S. Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. *Proc. Natl. Acad. Sci. USA* 88, 7844-7848 (1991).
 22. Bull, H.A., Brickell, P.M. & Dowd, P.M. Src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells. *FEBS Lett.* 351, 41-44 (1994).
 23. Tolma, S.S. et al. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J. Cell Biol.* 122, 497-511 (1993).
 24. Coullich, S. & Clarke, P. Apoptosis: does stress kill? *Curr. Biol.* 6, 1586-1588 (1996).
 25. Le-Niculescu, H. et al. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol. Cell Biol.* 19, 751-763 (1999).
 26. Verheij, M. et al. Requirement for ceramide-initiated SAPK/JNK signaling in stress-induced apoptosis. *Nature* 380, 75-79 (1996).
 27. Ichijo, H. et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275, 90-94 (1997).
 28. Kumar, S. et al. Activation of the HIV-1 long terminal repeat by cytokines and environmental stress requires an active CSBP/p38 MAP kinase. *J. Biol. Chem.* 271, 30864-30869 (1996).
 29. Polverini, P.J., Bouck, N.P. & Rastinejad, F. Assay and purification of naturally occurring inhibitor of angiogenesis. *Meth. Enzymol.* 198, 440-450 (1991).
 30. Schultz-Cherry, S. & Murphy-Ullrich, J.E. Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *J. Cell Biol.* 122, 923-932 (1993).
 31. Storgard, C.M. et al. Decreased angiogenesis and arthritic disease in rabbits treated with an $\alpha\beta 3$ antagonist. *J. Clin. Invest.* 103, 47-54 (1999).
 32. Jacobson, M.D., Well, M. & Raff, M.C. Programmed cell death in animal development. *Cell* 88, 347-354 (1997).
 33. Dragovich, T., Rudin, C.M. & Thompson, C.B. Signal transduction pathways that regulate cell survival and cell death. *Oncogene* 17, 3207-3213 (1998).
 34. Nor, J.E., Christensen, J., Mooney, D.J. & Polverini, P.J. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *Am. J. Pathol.* 154, 375-384 (1999).
 35. Benjamin, L.E., Goljari, D., Kim, A., Pade, D. & Keshet, E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J. Clin. Invest.* 103, 159-165 (1999).
 36. Gupta, K. et al. VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. *Exp. Cell Res.* 247, 495-504 (1999).
 37. Erpel, T. & Courtneidge, S.A. Src family protein tyrosine kinases and cellular signal transduction pathways. *Curr. Opin. Cell Biol.* 7, 176-182 (1995).
 39. Miyakawa, T. et al. Fyn-kinase as a determinant of ethanol sensitivity: relation to NMDA-receptor function. *Science* 278, 698-701 (1997).
 39. Grant, S.G.N. et al. Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science* 258, 1903-1910 (1992).
 40. Juo, P., et al. Fas activation of the p38 mitogen-activated protein kinase signaling pathway requires ICE/CED-3 family proteases. *Mol. Cell Biol.* 17, 24-35 (1997).
 41. Cardone, M.H., Salvesen, G.S., Widmann, C., Johnson, G. & Frisch, S.M. The regulation of anolids: MEKK-1 activation requires cleavage by caspases. *Cell* 90, 315-323 (1997).
 42. Kothakota, S. et al. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278, 294-298 (1997).
 43. Salazar, R., Bell, S.E. & Davis, G. E. Coordinate induction of the actin cytoskeletal regulatory proteins gelsolin, vasodilator-stimulated phosphoprotein, and profilin during capillary morphogenesis *in vitro*. *Exp. Cell Res.* 249, 22-32 (1999).
 44. Brooks, P.C. et al. Integrin $\alpha V \beta 3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 79, 1157-1164 (1994).
 45. Stromblad, S., Becker, J.C., Yebra, M., Brooks, P.C. & Chersesh, D.A. Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin $\alpha V \beta 3$ during angiogenesis. *J. Clin. Invest.* 98, 426-433 (1996).
 46. Streilein, J.W. Unraveling immune privilege. *Science* 270, 1158-1159 (1995).
 47. Schlaepfer, D.D. & Hunter, T. Evidence for *in vivo* phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol. Cell Biol.* 16, 5623-5633 (1996).
 48. Febrailo, M., Abumrad, N.A., Haljar, D.P., Sharma, K., Pearce, S.F.A. & Silverstein, R.L. A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. *J. Biol. Chem.* 274, 19055-19062 (1999).

EXHIBIT C

Targeted Overexpression of the Angiogenesis Inhibitor Thrombospondin-1 in the Epidermis of Transgenic Mice Prevents Ultraviolet-B-Induced Angiogenesis and Cutaneous Photo-Damage

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Chronic ultraviolet-B irradiation of the skin results in epidermal hyperplasia, degradation of extracellular matrix molecules, and formation of wrinkles. To characterize the biologic role of the vascular system in the mediation of ultraviolet-B-induced skin damage, we performed quantitative analyses of cutaneous blood vessels of mice after 10 wk of ultraviolet-B irradiation. Skin vascularization was greatly increased after chronic ultraviolet-B exposure with a significant increase of both the number and the size of dermal blood vessels, associated with upregulation of vascular endothelial growth factor expression in the hyperplastic epidermis. To directly study whether inhibition of angiogenesis may diminish ultraviolet-B-induced cutaneous damage, wild-type and transgenic mice with skin-specific overexpres-

sion of the endogenous angiogenesis inhibitor thrombospondin-1 were subjected to the same ultraviolet-B irradiation regimen. Ultraviolet-B-irradiated thrombospondin-1 transgenic mice showed a significantly reduced skin vascularization, decreased endothelial cell proliferation, and increased endothelial cell apoptosis rates, compared with wild-type mice. Moreover, dermal photo-damage and wrinkle formation were greatly reduced in thrombospondin-1 transgenic mice. These results reveal an important role of the cutaneous vascular system in mediating ultraviolet-B-induced skin damage and suggest inhibition of angiogenesis as a potential new approach for the prevention of chronic cutaneous photo-damage. **Key words:** VEGF/skin aging/endothelium. *J Invest Dermatol* 118:800-805, 2002

Skin alterations observed after a single exposure to ultraviolet-B (UVB) irradiation (290-320 nm wavelength) include erythema (Cox *et al*, 1992; Kripke, 1994), vascular hyperpermeability, dilation of dermal blood vessels, and epidermal hyperplasia (Pearse *et al*, 1987; Berton *et al*, 1997). Prolonged sun exposure of human skin results in wrinkle formation, elastosis, and degradation of matrix macromolecules (Kligman, 1989; Leyden *et al*, 1989), and leads to an enhanced risk for the development of epithelial skin cancers (Kripke, 1994). The pronounced vascular changes observed after acute UVB irradiation suggest that the cutaneous vasculature plays an important role in the mediation of acute photo-damage. Several angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and interleukin-8 (Kramer *et al*, 1993; Strickland *et al*, 1997; Bielenberg *et al*, 1998) have been found upregulated after acute UVB irradiation of the skin, whereas a decreased expression of interferon- β , a cytokine with antiangiogenic activity, has been reported (Bielenberg *et al*, 1998). The biologic role of skin blood vessels in the pathogenesis of chronic UV damage has remained unclear, however.

Angiogenesis, the formation of new blood vessels from pre-existing vessels, involves increased microvascular permeability, degradation of extracellular matrix molecules, and proliferation and migration of endothelial cells, leading to the formation of new capillaries. In normal skin, angiogenesis is restricted to the perifollicular vasculature during the growth phase of the hair follicle (Yano *et al*, 2001). The skin can initiate a rapid angiogenic response during wound healing and inflammation, however. We have previously identified VEGF, released by epidermal keratinocytes, as a major skin angiogenesis factor (Detmar, 1996). VEGF expression is upregulated in the hyperplastic epidermis of psoriasis (Detmar *et al*, 1994), in healing wounds (Brown *et al*, 1992), and in other skin diseases characterized by enhanced angiogenesis (Brown *et al*, 1995a; 1995b). Moreover, targeted overexpression of VEGF in the epidermis of transgenic mice resulted in enhanced skin vascularization with increased numbers of tortuous and hyper-permeable blood vessels (Detmar *et al*, 1998). In contrast, much less is known about the functional role of endogenous inhibitors of angiogenesis in the skin. Thrombospondin-1 (TSP-1) is a 450 kDa matricellular protein that inhibits proliferation and migration of endothelial cells *in vitro* and potently diminishes squamous cell carcinoma growth and angiogenesis *in vivo* (Tolsma *et al*, 1993; Bleuel *et al*, 1999; Streit *et al*, 1999). In normal human skin, TSP-1 mRNA is produced by basal epidermal keratinocytes and TSP-1 protein is deposited in the basement membrane area (Wight *et al*, 1985), contributing to the normal antiangiogenic barrier that

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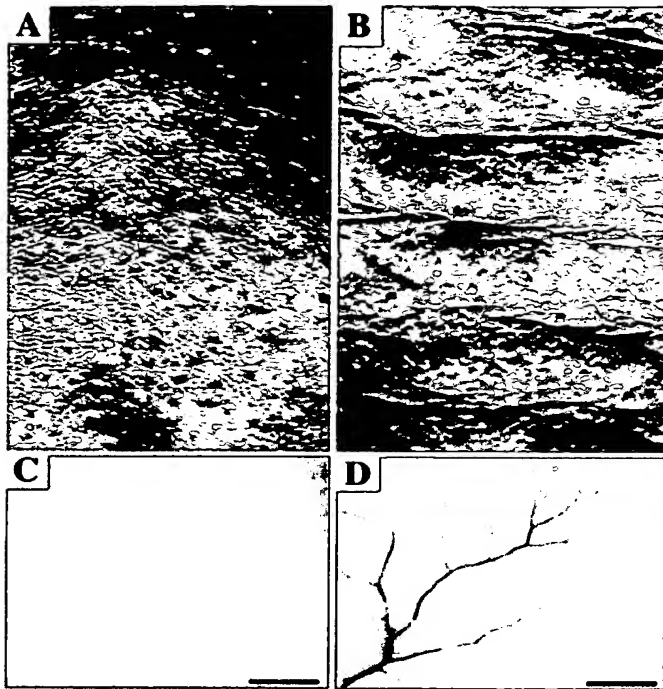


Figure 1. Wrinkle formation and enhanced skin vascularization after long-term UVB irradiation. Skin replicas of mice treated with long-term UVB irradiation (B) reveal wrinkle formation and textural damage, compared with nonirradiated skin (A). Increased cutaneous vascularization with prominent enlargement of blood vessels in chronically UVB-treated skin (D), compared with control skin (C). Panels C and D depict the underside of the skin. Scale bar: 3 mm.

separates the avascular epidermis from the vascularized dermis (Detmar, 2000).

We hypothesized that endothelial cell activation and induction of skin angiogenesis might play a critical role in the mediation of extracellular matrix degradation that is characteristic for chronic UVB damage. Therefore, we first performed a quantitative analysis of cutaneous blood vessels after repeated UVB exposures, using an established mouse model for UVB-induced chronic skin damage that has been previously characterized in detail (Bissett *et al.*, 1987). Similar to the findings in humans, long-term UVB irradiation of mouse skin leads to epidermal hyperplasia, elastosis, and degradation of the extracellular matrix (Kligman *et al.*, 1984; Bissett *et al.*, 1987; Lavker and Kligman, 1988). In a next step, we then investigated whether inhibition of skin angiogenesis might prevent UVB-induced skin damage, using transgenic mice with skin-specific overexpression of the angiogenesis inhibitor TSP-1 (Streit *et al.*, 2000). The phenotype of these mice includes decreased angiogenesis and granulation tissue formation during tissue repair, whereas no major abnormalities were found in normal, non-wounded skin (Streit *et al.*, 2000). Here, we report that chronic UVB irradiation results in pronounced cutaneous angiogenesis and in upregulation of VEGF expression by epidermal keratinocytes. Targeted overexpression of the angiogenesis inhibitor TSP-1 in the epidermis of transgenic mice inhibited UVB-induced angiogenesis, cutaneous photo-damage, and wrinkle formation.

MATERIALS AND METHODS

UVB irradiation regimen In a first experiment, 8-wk-old female hairless Skh-1 mice ($n = 7$ per group) were exposed to UVB irradiation, using a bank of four equally spaced fluorescent lamps (Southern New

England Ultraviolet, ME) (Kochevar *et al.*, 1993). The height of the lamps was adjusted to deliver 0.35 mW per cm^2 at the dorsal skin surface of the mice. Mice were irradiated with UVB three times weekly for 10 wk, with a starting dose of 0.5 minimal erythema dose (MED) (20 mJ per cm^2) and gradual increases in increments of 0.5 MED to a maximum dose of 4.5 MED (Kligman, 1989). The total cumulative dose of UVB was 5.62 J per cm^2 . No acute sunburn reactions were observed. Control mice were sham-irradiated. In an additional experiment, 8-wk-old female K14/TSP-1 transgenic mice (Streit *et al.*, 2000) or FVB wild-type controls ($n = 7$ per group) were treated with UVB irradiation as described above for a total of 12 wk (cumulative UVB dose 6.52 J per cm^2). After 12 wk, mice were sacrificed and skin replicas were obtained using silicon rubber (SILFLO; Flexico Developments, U.K.) as described previously (Chen *et al.*, 1992). Back skin samples were either snap-frozen in liquid nitrogen or fixed in 10% formaldehyde. All animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Immunohistochemistry for CD31 and computer-assisted morphometric vessel analysis Immunohistochemical stainings were performed on $7 \mu\text{m}$ frozen sections as described previously (Streit *et al.*, 2000), using a monoclonal rat antimouse CD31 antibody (Pharmingen, San Diego, CA). Representative sections were obtained from five UVB-irradiated mice of each experimental group and from five age-matched, non-UVB-irradiated control mice, and were analyzed using a Nikon E-600 microscope (Nikon; Melville, NY). Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), and morphometric analyses were performed using the IP-LAB software (Scanalytics, Fairfax, VA) as described previously (Streit *et al.*, 2000). Three different fields of each section were examined at $60\times$ magnification, and the number of vessels per mm^2 , the average vessel size, and the relative area occupied by blood vessels were determined in the dermis, in an area within $100 \mu\text{m}$ distance from the epidermal-dermal junction. The two-sided unpaired Student's *t* test was used to analyze differences in microvessel density and vascular size. In addition, paraffin sections were obtained from the skin of the same mice, and routine hematoxylin-eosin stains and LUNA stains were performed as described previously (Prophet *et al.*, 1992).

Proliferation and apoptosis assays To analyze endothelial cell proliferation, double immunofluorescent stainings for the endothelial cell marker CD31 and the proliferation marker Ki-67 (Gerdes *et al.*, 1983) were performed on $7 \mu\text{m}$ frozen sections, using a monoclonal rat antimouse CD31 antibody and a rabbit anti-Ki-67 polyclonal antibody (Novocastra Laboratories, Burlingame, CA). Antirat IgG conjugated with FITC and antirabbit IgG conjugated with Texas-Red (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies. Representative sections were obtained from five mice for each experimental group and were analyzed using a Nikon E-600 microscope. Digital images of CD31 (green) and Ki-67 (red) stains were obtained in identical fields and were combined to reveal proliferative endothelial cells (yellow). Apoptotic endothelial cells were detected by double immunofluorescence, using the Fluorescence-FragEL DNA fragmentation detection kit (Oncogene, Cambridge, MA) and an antimouse CD31 antibody together with an antirat IgG conjugated with Texas-Red as described previously (Streit *et al.*, 2000).

In situ hybridization *In situ* hybridization was performed on paraffin sections as described earlier (Detmar *et al.*, 1998). Briefly, slides were processed through xylene to remove paraffin, and then passed sequentially through graded alcohols; 0.2 M HCl ; Tris/ethylenediamine tetraacetic acid (EDTA) with $3 \mu\text{g per ml}$ proteinase K; 0.2% glycine; 4% paraformaldehyde in phosphate-buffered saline pH 7.4; 0.1 M triethanolamine containing $1/200$ (vol/vol) acetic anhydride; and $2\times$ sodium citrate/chloride buffer (SSC). Slides were hybridized overnight at 52°C with ^{35}S -labeled riboprobes in the following mixture: 0.3 M NaCl , $0.01 \text{ M Tris pH 7.6}$, 5 mM EDTA , 50% formamide, 10% dextran sulfate, 0.1 mg per ml yeast tRNA, and 0.01 M dithiothreitol. Post-hybridization washes included $2\times \text{SSC}/50\%$ formamide/ 10 mM dithiothreitol at 65°C and $2\times \text{SSC}$. Slides were then dehydrated through graded alcohol containing 0.3 M ammonium acetate, dried, coated with Kodak NTB2 emulsion, and stored in the dark at 4°C for 2 wk. The emulsion was developed with Kodak 19 developer and the slides were counterstained with hematoxylin. Antisense and sense single-stranded ^{35}S -labeled RNA probes for VEGF were prepared from a 393 bp rat VEGF cDNA fragment (Detmar *et al.*, 1998), cloned into pGEM-3Z (Promega).

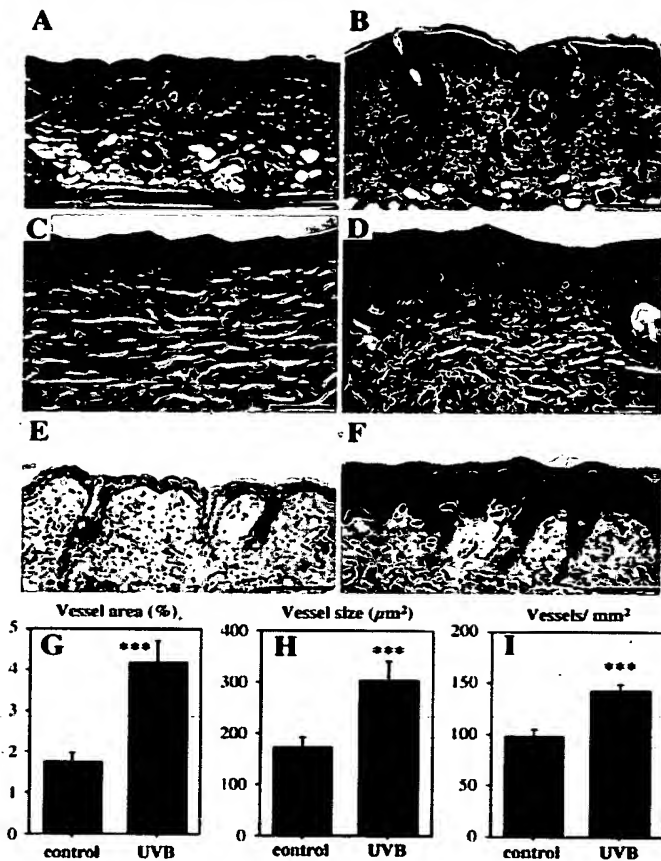


Figure 2. Cutaneous photodamage and increased vascularization after long-term UVB irradiation. Thickening of epidermis and dermis and increased inflammatory cell infiltration in the skin of mice treated with long-term UVB irradiation (B), compared with nonirradiated control skin (A). Hematoxylin-eosin stains and LUNA stains demonstrate irregular organization of elastic and collagen fibers in the papillary dermis of UVB-treated skin (D), compared with control skin (C). CD31 immunostains reveal increased vascularization with more numerous and enlarged blood vessels in UVB-treated mice (F), most prominently in the upper dermis, compared with control mice (E). Computer-assisted morphometric analysis of CD31-stained skin sections revealed a significant increase of the average vessel size (H), vessel density (I), and the relative skin area covered by vessels (G) in UVB-irradiated mice, compared with nonirradiated controls. Data are expressed as mean \pm SD of three independent experiments. *** $p < 0.001$ (increase over non-UVB-irradiated). Scale bars: (A, B, E, F) 150 μm , (C, D) 100 μm .

RESULTS

Enhanced skin angiogenesis after long-term UVB irradiation After 10 wk of UVB irradiation (cumulative dose 5.65 J per cm^2), replicas were obtained from the back skin of UVB-irradiated and of nonirradiated mice in order to evaluate the skin surface relief as a parameter for the extent of skin damage. Pronounced formation of wrinkles was observed in UVB-irradiated mice (Fig 1B), whereas no visible wrinkles were detected in nonirradiated control mice (Fig 1A). Macroscopic examination of the underside of the skin demonstrated increased subcutaneous vascularization in UVB-irradiated mice with prominent enlargement of blood vessels (Fig 1C, D).

Histologic analysis showed thickening of the epidermis, dermis, and sebaceous glands (Lesnik *et al*, 1992) in UVB-treated mice,

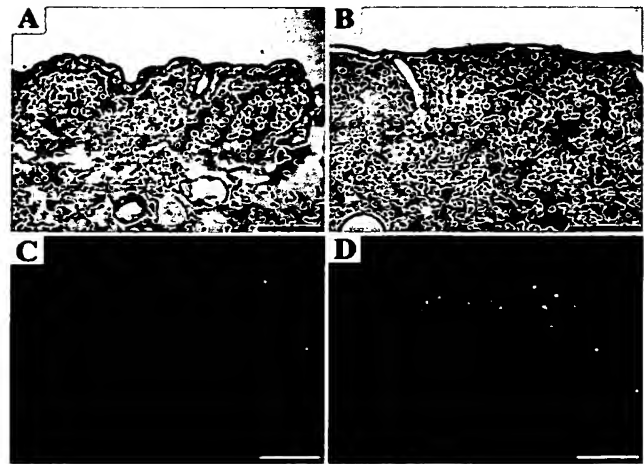


Figure 3. Enhanced epidermal VEGF expression after long-term UVB irradiation. *In situ* hybridization demonstrates enhanced epidermal VEGF mRNA expression in suprabasal epidermal keratinocytes after chronic UVB irradiation (B, D), compared with little or no VEGF mRNA expression in nonirradiated skin (A, C). Bright-field (A, B) and dark-field (C, D) microscopy. Scale bar: 150 μm .

accompanied by accumulation of inflammatory cells in the upper dermis (Fig 2A, B). Moreover, we found fragmented and less organized collagen fibers and elastic fibers in UVB-irradiated skin (Fig 2D) compared with the regular pattern observed in nonirradiated control skin (Fig 2C). Immunostains for CD31 revealed an increased number of enlarged blood vessels in the dermis of UVB-irradiated mice (Fig 2F) compared with untreated controls (Fig 2E). These changes were most prominent in the papillary dermis, in an area immediately underlying the epidermis (Fig 2F).

We next performed quantitative, computer-assisted morphometric analyses of cutaneous vessel density and size, using CD31-stained tissue sections. Chronic UVB irradiation resulted in a significant ($p < 0.001$) increase in vascular density compared with nonirradiated controls (Fig 2I). Vessels in UVB-irradiated skin were also significantly larger ($p < 0.001$) with a 67% increase in size (Fig 2H), leading to a more than 130% increase ($p < 0.001$) in the cutaneous area covered by vessels (Fig 2G).

Enhanced epidermal VEGF expression after long-term UVB irradiation Because we had previously identified VEGF as a potent inducer of skin angiogenesis in conditions associated with epidermal hyperplasia and increased dermal vascularity, we next examined the effect of long-term UVB irradiation on cutaneous VEGF mRNA expression. Using *in situ* hybridization, we found that VEGF mRNA expression was potently upregulated in the hyperplastic epidermis after long-term UVB irradiation (Fig 3B, D), whereas only sparse VEGF expression was found in the dermis. Little or no VEGF mRNA expression was detected in the skin of non-UVB-irradiated mice (Fig 3A, C).

Overexpression of TSP-1 inhibits UVB-induced cutaneous damage, wrinkle formation, and angiogenesis To investigate the functional significance of cutaneous angiogenesis for mediating the damaging effects of long-term UVB irradiation on the skin, we next subjected transgenic mice with skin-specific overexpression of the endogenous angiogenesis inhibitor TSP-1 to the same UVB irradiation regimen. These mice have been previously characterized and show potent inhibition of wound-induced angiogenesis (Streit *et al*, 2000). After 10 wk of UVB irradiation (cumulative UVB dose 6.52 J per cm^2), all wild-type mice showed formation of wrinkles on their dorsal skin (Fig 4A). In contrast, no wrinkle formation was observed in TSP-1

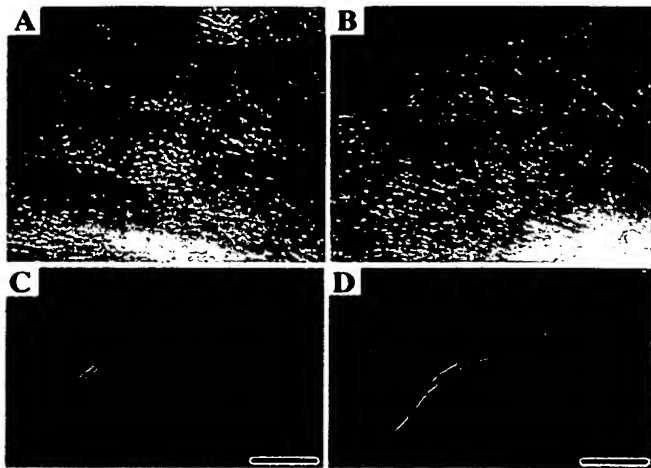


Figure 4. Reduced wrinkle formation and diminished cutaneous vascularization in K14/TSP-1 transgenic mice after long-term UVB irradiation. Skin replicas (A, B) and macroscopic view of the underside of the skin (C, D) demonstrate reduced wrinkle formation (B) and diminished cutaneous vascularization, with a predominant effect on small-sized vessels (D) in K14/TSP-1 transgenic mice, compared with wild-type littermates (A, C).

overexpressing transgenic mice (Fig 4B). Macroscopically, K14/TSP-1 transgenic mice also showed reduced skin vascularization (Fig 4D) compared with wild-type littermates (Fig 4C).

Histologic analysis revealed that the UVB-induced thickening of the dermis and the subcutis was less pronounced in K14/TSP-1 transgenic mice (Fig 5B) compared with wild-type mice (Fig 5A), whereas we did not find any major differences in the thickness of the epidermis. We also detected a reduction of inflammatory cell infiltration and a more regular arrangement and structure of collagen and elastic fibers in the dermis of K14/TSP-1 transgenic mice (Fig 5B, D) mice compared with wild-type mice (Fig 5A, C). Moreover, the skin vascularity was greatly reduced in K14/TSP-1 transgenic mice (Fig 5E, F). Morphometric analysis of CD31-stained skin sections, obtained after 12 wk of UVB irradiation, showed a more than 55% reduction of the average vessel size in TSP-1 transgenic mice compared with wild-type mice (Fig 5H; $p < 0.001$), and a significant reduction in the cutaneous area covered by vessels (Fig 5G; $p < 0.001$), whereas vessel density (number of vessels per area unit) was only slightly reduced (Fig 5J). In contrast, no significant differences of the average area occupied by vessels (Fig 5J), the average vessel size (Fig 5K), or the vessel density (Fig 5L) were detected in the nonirradiated skin of age-matched TSP-1 transgenic and wild-type mice. Double immunofluorescent stainings for CD31 and Ki-67 demonstrated a marked reduction in the number of proliferating endothelial cells in the dermis of UVB-irradiated TSP-1 transgenic mice (Fig 6B), compared with UVB-irradiated wild-type littermates (Fig 6A). Moreover, TUNEL assays, combined with CD31 stains, revealed an increase of endothelial cell apoptosis in the skin of TSP-1 transgenic mice (Fig 6D) compared with wild-type littermates (Fig 6C).

DISCUSSION

Photoaged skin is characterized by epidermal hyperplasia, dermal elastosis, and matrix protein degradation (Kligman *et al*, 1986; Leyden *et al*, 1989), and by the presence of perivascular lymphohistocytic dermal infiltrates (Lavker and Kligman, 1988). Whereas previous studies found vascular activation including endothelial cell proliferation after acute UVB irradiation of the skin (Bielenberg *et al*, 1998), the distinct effects of chronic UVB

exposure on cutaneous vascularization and the biologic importance of dermal blood vessels for the mediation of chronic UVB-induced cutaneous damage have remained unknown. Our results reveal that chronic UVB irradiation of the skin is associated with pronounced dermal angiogenesis and with increased VEGF expression in the hyperplastic epidermis, and that targeted inhibition of skin angiogenesis by TSP-1 diminishes UVB-induced dermal damage and wrinkle formation.

After 10 wk of UVB irradiation of Skh-1 hairless mice, an established experimental model for chronic photoaging (Kligman, 1989), we detected wrinkle formation and the characteristic histologic features of epidermal and dermal thickening, associated with increased detection of disorganized elastic and collagen fibers in the dermis. Quantitative image analysis of tissue sections stained for the endothelial junction molecule CD31 (Dejana *et al*, 1995) revealed a marked induction of skin angiogenesis after long-term UVB irradiation, with a significant increase of both vessel density and vessel size. These vascular changes were comparable to the angiogenic changes that occur during cutaneous wound healing where both sprouting of preexisting blood vessels and vessel enlargement contribute to the formation of the vessel-rich granulation tissue (Streit *et al*, 2000). In contrast, chronic inflammatory skin diseases such as psoriasis predominantly show vascular remodeling with elongation and enlargement of cutaneous microvessels but without the formation of new vessel sprouts. Our findings indicate that chronic UVB irradiation of the skin results in a chronic tissue-repair-like reaction, and they suggest that angiogenesis might play an important role in the mediation of chronic UVB-induced skin damage.

VEGF is a major, keratinocyte-derived skin angiogenesis factor (Detmar *et al*, 1995) with increased expression in the hyperplastic epidermis of lesional psoriatic skin (Detmar *et al*, 1994) and of other skin diseases associated with dermal angiogenesis (Brown *et al*, 1995a; 1995b), as well as in the neo-epidermis of healing wounds (Brown *et al*, 1992; Kishimoto *et al*, 2000). In general, VEGF has been found to be upregulated in skin conditions that are characterized by epidermal hyperplasia and dermal angiogenesis. We therefore hypothesized that epidermis-derived VEGF might also play a major role in the mediation of chronic UVB-induced skin angiogenesis. Indeed, we found a strong upregulation of VEGF mRNA expression in the hyperplastic epidermis, but not in the dermis, of chronically UVB-irradiated skin. These findings are in accordance with previous reports that acute UVB irradiation induced VEGF expression in human epidermal keratinocytes *in vitro* (Brauchle *et al*, 1996) and *in vivo* (Bielenberg *et al*, 1998). The exact mechanisms of UVB-induced VEGF expression remain to be established. Increasing evidence, however, suggests that several growth factors that mediate the characteristic epidermal hyperplasia, including transforming growth factor α and keratinocyte growth factor (Detmar *et al*, 1995; Brauchle *et al*, 1996; Gille *et al*, 1997), can act to enhance epidermal VEGF gene expression.

To directly investigate whether skin angiogenesis may play a direct biologic role in the mediation of chronic UVB-induced skin damage, we next subjected transgenic mice with skin-specific overexpression of the angiogenesis inhibitor TSP-1 to chronic UVB irradiation. Using an established keratin 14 (K14) promoter cassette to target TSP-1 transgene expression to epidermal keratinocytes, we have established K14/TSP-1 transgenic mice that are characterized by increased levels of epidermal TSP-1 expression, by normal thickness and morphology of the epidermis and dermis, and by potent inhibition of skin angiogenesis during cutaneous wound healing (Streit *et al*, 2000). The use of the K14 promoter ensures high transgene expression under conditions of epidermal hyperplasia because K14 gene expression is greatly enhanced in proliferating keratinocytes. Our results reveal that epidermal overexpression of TSP-1 inhibited dermal photodamage and collagen and elastic fiber disorganization, and also prevented the formation of skin wrinkles. This effect was associated with a potent inhibition of skin angiogenesis, with decreased endothelial proliferation rates and with increased apoptosis of endothelial cells.

In fact, the skin vascularity (vessel density, vessel size) in UVB-irradiated TSP-1 transgenic mice was comparable to the extent of skin vascularity observed in age-matched nonirradiated wild-type and TSP-1 transgenic mice, indicating that the vascular response to UVB irradiation was completely blunted by TSP-1. It is of interest that both baseline vascularity and the UVB-induced angiogenesis were higher in the skin of FVB mice compared with Skh-1 mice, confirming previous results regarding the heterogeneity of the angiogenic response in different mouse strains (Rohan *et al*, 2000).

It has been reported that TSP-1 mediates inhibition of angiogenesis by specific interactions of distinct sequences within

the type 1 repeats with the CD36 receptor on endothelial cells, resulting in enhanced endothelial cell apoptosis rates (Jimenez *et al*, 2000). Recent evidence suggests that TSP-1 also inhibits the activation of matrix metalloproteinase-2 (MMP-2) and MMP-9, with potential implications for its antiangiogenic effects and its inhibitory activity on wound granulation tissue formation (Bein and Simons, 2000; Tarabozetti *et al*, 2000; Rodriguez-Manzanique *et al*, 2001). It is of interest that inhibition of the UVB-induced activation of MMP-9 activity has also been implicated as a mechanism of action of topical tretinoin treatment (Kang, 1998). Therefore, in addition to its potent antiangiogenic activity, effects of TSP-1 on the activation of MMPs or on the activation of TGF- β (Bornstein, 2001) might also have contributed to the reduction of UVB-induced skin damage. The relative importance of these distinct mechanisms for the multiple *in vivo* functions of TSP-1 remain to be established in future studies.

TSP-1 overexpression potentially reduced the size of angiogenic blood vessels whereas no major reduction of the vascular density was detected. These results are in accordance with the predominant inhibition of vascular enlargement in the wound granulation tissue in TSP-1 overexpressing transgenic mice (Streit *et al*, 2000), and

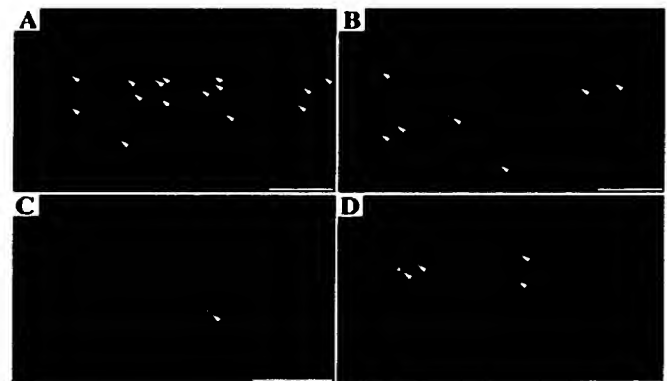
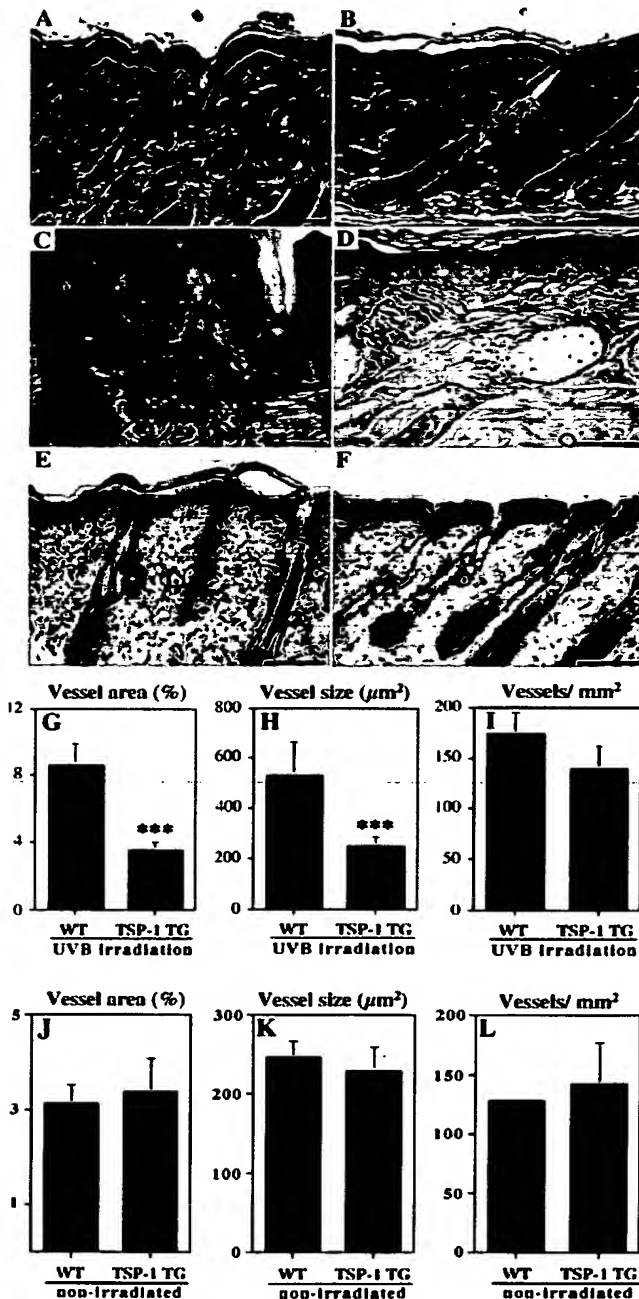


Figure 6. Reduced endothelial proliferation and increased endothelial apoptosis in TSP-1 transgenic mice. Differential immunofluorescent stains for CD31 (green) and Ki67 (red) demonstrate reduced numbers of proliferating endothelial cells (arrowheads) in K14/TSP-1 transgenic mice (B) after long-term UVB irradiation, compared with wild-type littermates (A). Scale bar: 150 μm . Double immunofluorescent stains for CD31 (red) and for apoptotic nuclei (green) reveal increased numbers of apoptotic endothelial cells (arrowheads) in UVB-irradiated K14/TSP-1 transgenic mice (D) compared with wild-type littermates (C). Scale bar: 250 μm .

Figure 5. Diminished dermal photo-damage and angiogenesis in TSP-1 transgenic mice. Hematoxylin-eosin (A, B) and LUNA (C, D) stains show reduced inflammatory cell infiltration and more regular arrangement and structure of collagen and elastic fibers in TSP-1 transgenic mice (B, D) after long-term UVB irradiation, compared with wild-type littermates (A, C). Elastic fibers are stained dark purple in (C) and (D). Scale bars: (A, B) 150 μm , (C, D) 100 μm . CD31 immunostains demonstrate diminished vascularization with reduced vessel sizes in the dermis of UVB-treated TSP-1 transgenic mice (F), compared with wild-type mice (E). Computer-assisted morphometric analysis of CD31-stained skin sections after 10 wk of UVB irradiation revealed a significant decrease of the average vessel size (H) and relative area occupied by vessels (G) in TSP-1 transgenic mice, whereas the vessel density was only slightly reduced (I). In contrast, no significant differences of the average area occupied by vessels (J), the average vessel size (K), or the vessel density (L) were detected in the nonirradiated skin of age-matched TSP-1 transgenic and wild-type mice. Data are expressed as mean \pm SD of three independent experiments. *** $p < 0.001$ (decrease compared with wild-type).

with our previous findings in human squamous cell carcinomas, in which transfected TSP-1 exerted a more potent inhibitory effect on peritumoral vessel sizes than on vessel numbers (Streit *et al*, 1999). Although the mechanisms for this preferential effect on vessel size remain unknown, increasing evidence suggests that the increased vessel size of angiogenic vessels appears to be a more sensitive target for angiogenesis inhibition. Indeed, treatment of human tumor xenografts with a neutralizing anti-VEGF antibody resulted in a rapid reduction of tumor vessel diameters (Yuan *et al*, 1996). Together, our results indicate that inhibition of the repair-associated, UVB-induced angiogenesis in mice also prevents dermal photo-damage including the formation of wrinkles.

The observed induction of skin angiogenesis by chronic UVB irradiation, together with the inhibition of cutaneous photo-damage and wrinkle formation by skin-specific overexpression of the angiogenesis inhibitor TSP-1, indicate that inhibition of skin angiogenesis might represent a potential new approach for the prevention of chronic cutaneous UVB damage. Because TSP-1 exerts multiple biologic functions, future studies with selective angiogenesis inhibitors, e.g., small molecule inhibitors of VEGF function, should provide more insight into the relative importance of the angiogenic response for UVB-induced cutaneous damage.

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REFERENCES

- Bein K, Simons M: Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *J Biol Chem* 275:32167-32173, 2000
- Berton TR, Mitchell DL, Fischer SM, Locniskar MF: Epidermal proliferation but not quantity of DNA photodamage is correlated with UV-induced mouse skin carcinogenesis. *J Invest Dermatol* 109:340-347, 1997
- Bielenberg DR, Bucana CD, Sanchez R, Donawho CK, Kripke ML, Fidler IJ: Molecular regulation of UVB-induced cutaneous angiogenesis. *J Invest Dermatol* 111:864-872, 1998
- Bissett DL, Hannon DP, Orr TV: An animal model of solar-aged skin: histological, physical, and visible changes in UV-irradiated hairless mouse skin. *Photochem Photobiol* 46:367-378, 1987
- Bluel K, Popp S, Fusenig NE, Stanbridge EJ, Boukamp P: Tumor suppression in human skin carcinoma cells by chromosome 15 transfer or thrombospondin-1 overexpression through halted tumor vascularization. *Proc Natl Acad Sci USA* 96:2065-2070, 1999
- Bornstein P: Thrombospondins as extracellular mediators of cell function. *J Clin Invest* 107:929-934, 2001
- Brauchle M, Funk JO, Kind P, Werner S: Ultraviolet B and H₂O₂ are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J Biol Chem* 271:21793-21797, 1996
- Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF, Van De Water L: Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med* 176:1375-1379, 1992
- Brown LF, Harnett TJ, Yeo K-T, et al: Increased expression of vascular permeability factor (vascular endothelial growth factor) in bullous pemphigoid, dermatitis herpetiformis, and erythema multiforme. *J Invest Dermatol* 104:744-749, 1995a
- Brown LF, Olbricht SM, Berse B, et al: Overexpression of vascular permeability factor (VPF/VEGF) and its endothelial cell receptors in delayed hypersensitivity skin reactions. *J Immunol* 154:2801-2807, 1995b
- Chen S, Kiss I, Tramsosch KM: Effects of all-trans retinoic acid on UVB-irradiated and non-irradiated hairless mouse skin. *J Invest Dermatol* 98:248-254, 1992
- Cox NH, Diffey BL, Farr PM: The relationship between chronological age and the erythral response to ultraviolet B radiation. *Br J Dermatol* 126:315-319, 1992
- Dejana E, Corada M, Lampugnani MG: Endothelial cell-to-cell junctions. *FASEB J* 9:910-918, 1995
- Detmar M: Molecular regulation of angiogenesis in the skin. *J Invest Dermatol* 106:207-208, 1996
- Detmar M: The role of VEGF and thrombospondins in skin angiogenesis. *J Dermatol Sci* 24:S78-S84, 2000
- Detmar M, Brown LF, Claffey KP, et al: Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med* 180:1141-1146, 1994
- Detmar M, Yeo K-T, Nagy JA, et al: Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. *J Invest Dermatol* 105:44-50, 1995
- Detmar M, Brown LF, Schön MP, et al: Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J Invest Dermatol* 111:1-6, 1998
- Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer* 31:13-20, 1983
- Gille J, Swerlick RA, Caughman SW: Transforming growth factor- α -induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. *EMBO J* 16:750-759, 1997
- Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N: Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med* 6:41-48, 2000
- Kang S: Photoaging and tretinoin. *Dermatol Clin* 16:357-364, 1998
- Kishimoto J, Ehama R, Ge Y, Kobayashi T, Nishiyama T, Detmar M, Burgeson RE: In vivo detection of human vascular endothelial growth factor promoter activity in transgenic mouse skin. *Am J Pathol* 157:103-110, 2000
- Kligman AM: The treatment of photoaged human skin by topical tretinoin. *Drugs* 38:1-8, 1989
- Kligman AM, Grove GL, Hirose R, Leyden JJ: Topical tretinoin for photoaged skin. *J Am Acad Dermatol* 15:836-859, 1986
- Kligman LH: The ultraviolet-irradiated hairless mouse: a model for photoaging. *J Am Acad Dermatol* 21:623-631, 1989
- Kligman LH, Duo CH, Kligman AM: Topical retinoic acid enhances the repair of ultraviolet damaged dermal connective tissue. *Connect Tissue Res* 12:139-150, 1984
- Kochevar IE, Moran M, Lyon N, Flotte T, Siebert E, Gange RW: Effects of systemic indomethacin, meclizine, and BW755C on chronic ultraviolet B-induced effects in hairless mouse skin. *J Invest Dermatol* 100:186-193, 1993
- Kramer M, Sachsenmaier C, Herrlich P, Rahmsdorf HJ: UV irradiation-induced interleukin-1 and basic fibroblast growth factor synthesis and release mediate part of the UV response. *J Biol Chem* 268:6734-6741, 1993
- Kripke ML: Ultraviolet radiation and immunology: something new under the sun - presidential address. *Cancer Res* 54:6102-6105, 1994
- Lavker RM, Kligman AM: Chronic heliodermatitis: a morphologic evaluation of chronic actinic dermal damage with emphasis on the role of mast cells. *J Invest Dermatol* 90:325-330, 1988
- Lesnik RH, Kligman LH, Kligman AM: Agents that cause enlargement of sebaceous glands in hairless mice. II. Ultraviolet radiation. *Arch Dermatol Res* 284:106-108, 1992
- Leyden JJ, Grove GL, Grove MJ, Thorne EG, Lufano L: Treatment of photodamaged facial skin with topical tretinoin. *J Am Acad Dermatol* 21:638-644, 1989
- Pearse AD, Gaskell SA, Marks R: Epidermal changes in human skin following irradiation with either UVB or UVA. *J Invest Dermatol* 88:83-87, 1987
- Prophet E, Mills B, Arrington J, Sobin L: *Laboratory Methods in Histotechnology* 1-236-237. Washington, DC: American Registry of Pathology, 1992
- Rodriguez-Manzanique JC, Lané TF, Ortega MA, Hynes RO, Lawler J, Iruela-Arispe ML: Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc Natl Acad Sci USA* 98:12485-12490, 2001
- Rohan RM, Fernandez A, Udagawa T, Yuan J, d'Amato RJ: Genetic heterogeneity of angiogenesis in mice. *FASEB J* 14:871-876, 2000
- Streit M, Velasco P, Brown LF, et al: Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am J Pathol* 155:441-452, 1999
- Streit M, Velasco P, Riccardi L, et al: Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. *EMBO J* 19:3272-3282, 2000
- Strickland I, Rhodes LE, Flanagan BF, Friedmann PS: TNF- α and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J Invest Dermatol* 108:763-768, 1997
- Tarabozetti G, Sonzogni L, Vergani V, et al: Posttranscriptional stimulation of endothelial cell matrix metalloproteinases 2 and 1 by endothelioma cells. *Exp Cell Res* 258:384-394, 2000
- Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N: Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J Cell Biol* 122:497-511, 1993
- Wight TN, Raugi GJ, Mumby SM, Bornstein P: Light microscopic immunolocalization of thrombospondin in human tissues. *J Histochem Cytochem* 33:295-302, 1985
- Yano K, Brown LF, Detmar M: Control of hair growth and follicle size by VEGF-mediated angiogenesis. *J Clin Invest* 107:409-417, 2001
- Yuan F, Chen Y, Dellian M, Safabakhsh N, Ferrara N, Jain RK: Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. *Proc Natl Acad Sci USA* 93:14765-14770, 1996

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(54) Title: METHODS OF PREVENTING UVB-INDUCED SKIN DAMAGE

(57) Abstract: The invention features a method preventing or treating long-term UVB-induced wrinkles in a subject. The method includes inhibiting angiogenesis in the skin of the subject.

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METHODS OF PREVENTING UVB-INDUCED SKIN DAMAGE

Related Applications

This application claims the benefit of U.S. Provisional application serial number 60/283,874, filed April 13, 2001, the contents of which are incorporated herein by reference in their entirety.

Background of the Invention

Photoaging due to chronic exposure to ultraviolet-B (UVB) irradiation results, *inter alia*, in the formation of wrinkles.

Summary of the Invention

The invention is based, in part, on the discovery that inhibition of skin angiogenesis can prevent UVB-induced skin damage, e.g., long term (chronic) UVB induced photoaging, e.g., wrinkle formation, *in vivo*, in mammals, e.g., humans.

Accordingly, the invention features a method preventing or treating long-term UVB-induced skin damage, e.g., wrinkles, in a subject. The method includes inhibiting angiogenesis in the skin of the subject. In a preferred embodiment, angiogenesis is inhibited before or at the time of a UVB exposure.

In a preferred embodiment, the method also includes identifying a subject, e.g., a mammal, e.g., a human or a non-human mammal, at risk of long term UVB-induced skin damage. The identification of a subject at risk for long term UVB-induced skin damage, e.g., wrinkles, can be performed e.g., by the subject, by a health care provider, or by a provider of cosmetics. The inhibition of angiogenesis can be performed, e.g., by the subject, by a health care provider, or by a provider of cosmetics.

In a preferred embodiment the subject is at least 5 years of age. Preferably, the subject is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, or more years of age.

In a preferred embodiment, wrinkle formation is prevented or reduced.

In a preferred embodiment, angiogenesis is inhibited by increasing the activity of one or more anti-angiogenic factors, e.g., increasing the activity of naturally occurring anti-angiogenic proteins such as TSP-2 or TSP-1 in the subject, thereby preventing wrinkle formation. TSP-2 activity can be increased, e.g., by administering an agent which increases a TSP-2 activity. In a preferred embodiment, an agent which increases a TSP-2 activity can be one or more of the following: a TSP-2 polypeptide, or a biologically active fragment or analog thereof, e.g., a TSP-2 derived polypeptide or retro-inverso polypeptide thereof; a nucleic acid encoding a TSP-2 polypeptide, or a biologically active fragment or analog thereof; an agonist of TSP-2, e.g., an antibody or a small molecule having or increasing TSP-2 activity; or an agent that increases TSP-2 nucleic acid expression, e.g., a small molecule which binds to the promoter region of TSP-2 and increases expression.

In a preferred embodiment, TSP-2 is increased by an agent, e.g., a small molecule, which induces TSP-2 expression. Examples of agents that can induce expression of TSP-2 include fetal calf serum and TGF- α . In preferred embodiments, an agent that induces TSP-2 expression is administered topically. In preferred embodiments, the agent is administered to a subject sufficiently before UVB exposure, e.g., sun exposure, such that an anti-angiogenesis effect is present in the subject's skin at the time of UVB exposure.

TSP-2 activity can also be increased by controlled delivery to the subject of a TSP-2 nucleic acid, or a TSP-2 protein, fragment, or analog. A TSP-2 nucleic acid, protein, fragment, or analog can be administered to the subject in combination with a controlled release device, e.g., a biocompatible polymer, micro particle, or mesh. The device can reduce degradation and control the release of the TSP-2 nucleic acid, protein, fragment, or analog. Such a TSP-2 biocompatible controlled release system can be administered to the subject, e.g., by injection or implantation, e.g., intramuscularly, subcutaneously, intravenously, or at an organ, joint cavity, or at a lesion.

The level of TSP-2 can also be increased by increasing the endogenous TSP-2 activity. Activity can be increased by increasing the level of expression of the gene, e.g.,

by increasing transcription of the TSP-2 gene; increasing the stability of the TSP-2 mRNA, e.g., by altering the secondary or tertiary structure of the mRNA; increasing the translation of TSP-2 mRNA, e.g., by altering the sequence of the TSP-2 mRNA; and/or increasing the stability of the TSP-2 protein. Transcription of the TSP-2 gene can be increased, e.g., by altering the regulatory sequences of the endogenous TSP-2 gene. In one embodiment the regulatory sequence can be altered by: the addition of a positive regulatory element (such as an enhancer or a DNA-binding site for a transcriptional activator); the deletion of a negative regulatory element (such as a DNA-binding site for a transcriptional repressor) and/or replacement of the endogenous regulatory sequence, or elements therein, with that of another gene, thereby allowing the TSP-2 gene to be transcribed more efficiently.

In a preferred embodiment, the agent is a compound, e.g., small molecule, which induces TSP-2.

TSP-1 activity can be increased, e.g., by administering an agent which increases a TSP-1 activity. In a preferred embodiment, an agent which increases a TSP-1 activity can be one or more of the following: a TSP-1 polypeptide, or a biologically active fragment or analog thereof, e.g., a TSP-1 derived polypeptide or retro-inverso polypeptide thereof; a nucleic acid encoding a TSP-1 polypeptide, or a biologically active fragment or analog thereof; an agonist of TSP-1, e.g., an antibody or a small molecule having or increasing TSP-1 activity; or an agent that increases TSP-1 nucleic acid expression, e.g., a small molecule which binds to the promoter region of TSP-1 and increases expression.

In a preferred embodiment, TSP-1 is increased by an agent, e.g., a small molecule, which induces TSP-1 expression. Examples of agents that can induce expression of TSP-1 include fetal calf serum and TGF- α . In preferred embodiments, an agent that induces TSP-1 expression is administered topically. In preferred embodiments, the agent is administered to a subject sufficiently before UVB exposure, e.g., sun exposure, such that an anti-angiogenesis effect is present in the subject's skin at the time of UVB exposure.

TSP-1 activity can also be increased by controlled delivery to the subject of a TSP-1 nucleic acid, or a TSP-1 protein, fragment, or analog. A TSP-1 nucleic acid, protein, fragment, or analog can be administered to the subject in combination with a controlled release device, e.g., a biocompatible polymer, micro particle, or mesh. The device can reduce degradation and control the release of the TSP-1 nucleic acid, protein, fragment, or analog. Such a TSP-1 biocompatible controlled release system can be administered to the subject, e.g., by injection or implantation, e.g., intramuscularly, subcutaneously, intravenously, or at an organ, joint cavity, or at a lesion.

The level of TSP-1 can also be increased by increasing the endogenous TSP-1 activity. Activity can be increased by increasing the level of expression of the gene, e.g., by increasing transcription of the TSP-1 gene; increasing the stability of the TSP-1 mRNA, e.g., by altering the secondary or tertiary structure of the mRNA; increasing the translation of TSP-1 mRNA, e.g., by altering the sequence of the TSP-1 mRNA; and/or increasing the stability of the TSP-1 protein. Transcription of the TSP-1 gene can be increased, e.g., by altering the regulatory sequences of the endogenous TSP-1 gene. In one embodiment the regulatory sequence can be altered by: the addition of a positive regulatory element (such as an enhancer or a DNA-binding site for a transcriptional activator); the deletion of a negative regulatory element (such as a DNA-binding site for a transcriptional repressor) and/or replacement of the endogenous regulatory sequence, or elements therein, with that of another gene, thereby allowing the TSP-1 gene to be transcribed more efficiently.

In a preferred embodiment, the agent is a compound, e.g., small molecule, which induces TSP-1.

In a preferred embodiment, the agent which increases the activity of one or more anti-angiogenic factors, e.g., by inducing the activity of a naturally occurring anti-angiogenic protein such as TSP-2 or TSP-1 is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the agent is administered using a suitable delivery vehicle. Preferably, the

agent is included in a composition for topical use, e.g., the composition is a gel, cream, or liquid. The composition can further include a cosmetic ingredient, e.g., a fragrance or a sunscreen, e.g., octyl methoxycinnamate, aminobenzoic acid, oxybenzone, padimate O, homosalate, or titanium dioxide. The composition can also include a plant extract, e.g., aloe extract, grape extract. The composition can also include a vitamin, e.g., a vitamin A, e.g., retinol; a vitamin C, e.g., L-ascorbic acid or L-ascorbic acid palmitate; a vitamin E, e.g., tocopherol acetate.

In a preferred embodiment, the agent is administered to the subject sufficiently before UVB exposure, e.g., sun exposure, such that an anti-angiogenesis effect is present at the time of UVB exposure.

In another preferred embodiment, administration of the agent that increases the activity of one or more anti-angiogenic factors, e.g., by inducing the activity of a naturally occurring anti-angiogenic protein such as TSP-2 or TSP-1, is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times over at least as many days. In a preferred embodiment, the agent is administered chronically. In a preferred embodiment, the agent is administered at least once a week, preferably 2, 3, 4, 5 times a week or daily for at least two weeks, preferably for at least 1, 2, 3, 4, 5, or 6 months. For example, the agent is administered periodically over 3-12 weeks, e.g., it is administered throughout the summer. In a preferred embodiment, the agent is administered to and wrinkles are inhibited or prevented on one or more of: the subject's face, neck, chest, ears, hands, bald spots of the scalp, or any other skin that is exposed to UVB radiation.

In a preferred embodiment, the subject has been, or will be, exposed to long term UVB radiation.

In a preferred embodiment, the subject shows one or more signs of photoaging, e.g., wrinkles, lines, sagging, freckles, tanned skin, discoloration, hyperpigmentation, age spots, e.g., "liver spots", thinning of the skin, cataracts, epidermal hyperplasia, skin elastosis, degradation of extracellular matrix, or precancerous or cancerous skin growths (actinic keratoses, solar keratoses).

In a preferred embodiment, angiogenesis is inhibited by decreasing VEGF activity in the subject, e.g., by inhibiting signaling through the VEGF receptor, e.g., through KDR; by inhibiting the level of VEGF protein; decreasing the levels of VEGF gene expression; and/or decreasing VEGF protein production and/or activity, in the subject, thereby preventing UVB-induced skin damage, e.g., long term-UVB induced skin damage, e.g., wrinkle formation.

In a preferred embodiment, VEGF is inhibited by administering an agent which inhibits VEGF activity. An agent which inhibits VEGF activity can be one or more of: an agent, e.g., a small molecule, that inhibits a VEGF receptor, e.g., by inhibiting binding of VEGF to its receptor or by inhibiting VEGF receptor signaling; a VEGF nucleic acid molecule which can bind to a cellular VEGF nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein, e.g., an antisense molecule or VEGF ribozyme; an antibody that specifically binds to VEGF protein, e.g., an antibody that disrupts VEGF's ability to bind to its natural cellular target; an agent which decreases VEGF gene expression, e.g., a small molecule which binds the promoter of VEGF.

In another preferred embodiment, VEGF activity is inhibited by decreasing the level of expression of an endogenous VEGF gene, e.g., by decreasing transcription of the VEGF gene. In a preferred embodiment, transcription of the VEGF gene can be decreased by: altering the regulatory sequences of the endogenous VEGF gene, e.g., by the addition of a negative regulatory sequence (such as a DNA-binding site for a transcriptional repressor).

In another preferred embodiment, the agent is a compound, e.g., a small molecule, which inhibits VEGF activity, e.g., by inhibiting VEGF receptor signaling, or by interacting, directly or indirectly, with a VEGF promoter.

In a preferred embodiment, the agent which inhibits VEGF expression is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the agent is administered using a suitable delivery vehicle. Preferably, the agent is included in a composition for topical use, e.g.,

the composition is a gel, cream, or liquid. The composition can further include a cosmetic ingredient, e.g., a fragrance or a sunscreen, e.g., octyl methoxycinnamate, aminobenzoic acid, oxybenzone, padimate O, homosalate, or titanium dioxide. The composition can also include a plant extract, e.g., aloe extract, grape extract. The composition can also include a vitamin, e.g., a vitamin A, e.g., retinol; a vitamin C, e.g., L-ascorbic acid or L-ascorbic acid palmitate; a vitamin E, e.g., tocopherol acetate. In a preferred embodiment, an agent that inhibits VEGF expression is administered topically. In a preferred embodiment, the agent is applied sufficiently before UVB, e.g., sun, exposure such that an anti-angiogenesis effect is present at the time of UVB exposure.

In another preferred embodiment, administration of the agent is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times. In a preferred embodiment, the agent is applied chronically. In a preferred embodiment, the agent is applied at least once a week, preferably 2, 3, 4, 5 times a week or daily for at least two weeks, preferably for at least 1, 2, 3, 4, 5, or 6 months. For example, the agent is administered periodically over 3-12 weeks, e.g., it is administered throughout the summer. In a preferred embodiment, wrinkles are inhibited on: the subject's face, neck, chest, hands, or any other skin that has been exposed to UVB radiation.

In a preferred embodiment the method includes administering one or a more of an agent which increases TSP-2 activity, an agent which increases TSP-1 activity, or an agent which inhibits VEGF. In preferred embodiments, one or more inhibitors of angiogenesis, e.g., one or more agents that induce or increase an anti-angiogenesis inhibitor, are administered.

In another aspect, the invention features a method of preventing or treating UVB-induced skin damage, e.g., long term UVB-induced skin damage, e.g., wrinkles, in a subject. The method includes administering to the subject, e.g., topically, a composition comprising an inhibitor of angiogenesis, e.g., an agent, e.g., a small molecule, that increases or induces an inhibitor of angiogenesis, or an agent, e.g., a small molecule, that inhibits an angiogenic molecule, in an amount sufficient to reduce or prevent UVB-

induced skin damage, e.g., long term UVB-induced skin damage, e.g. wrinkles. In a preferred embodiment, the agent is administered sufficiently before UVB exposure, e.g., sun exposure, such that an anti-angiogenesis effect is present at the time of UVB exposure.

In a preferred embodiment, the agent is a compound, e.g., small molecule, which induces TSP-2.

In a preferred embodiment, the agent is a compound, e.g., a small molecule, that inhibits VEGF.

In a preferred embodiment, the agent is administered topically. The agent can be administered to the face, chest, ears, neck, hands, bald areas of the scalp, and other regions of the body. The treatment can involve more than one administration, e.g., at least two, three, or four administrations, of the angiogenesis inhibitor. The treatment can also involve daily administration of the angiogenesis inhibitor.

In a preferred embodiment, the inhibitor of angiogenesis, e.g. the agent that increases or induces the inhibitor of angiogenesis, is provided in a sterile composition.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-2.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-1.

In a preferred embodiment, the composition further includes a cosmetic ingredient, e.g., a fragrance, or a sunscreen, e.g., octyl methoxycinnamate, aminobenzoic acid, oxybenzone, padimate O, homosalate, or titanium dioxide. The composition can also include a plant extract, e.g., aloe extract, grape extract. The composition can also include a vitamin, e.g., a vitamin A, e.g., retinol; a vitamin C, e.g., L-ascorbic acid or L-ascorbic acid palmitate; a vitamin E, e.g., tocopherol acetate.

In a preferred embodiment, the composition is administered chronically. In a preferred embodiment, the composition is applied at least once a week, preferably 2, 3, 4, 5 times a week or daily for at least two weeks, preferably for at least 1, 2, 3, 4, 5, or 6 months. For example, the composition is applied throughout periodically over 3-12 weeks, e.g., throughout the summer. In a preferred embodiment the method includes administering one or a more of an agent which increases TSP-2 activity, an agent which

increases TSP-1 activity, or an agent which inhibits VEGF. In preferred embodiments one or more inhibitors of angiogenesis are administered.

In another aspect, the invention features a method of preventing UVB-induced skin damage, e.g., long term-UVB induced skin damage, e.g., a wrinkle, in a subject. The method includes identifying a subject in need of protection from UVB-induced skin damage, e.g., long term-UVB induced skin damage, e.g., protection from wrinkle formation; administering an inhibitor of angiogenesis, e.g., an agent, e.g., a small molecule, that increases or induces an inhibitor of angiogenesis or an agent, e.g., a small molecule, that inhibits an angiogenic molecule, to the subject; and evaluating the effect of the administration on wrinkle formation. The identification of a subject in need of protection from long term UVB-induced skin damage, e.g., wrinkles, can be performed e.g., by the subject, by a health care provider, or by a provider of cosmetics. The administration of an inhibitor of angiogenesis and the evaluation of the effect of the administration on wrinkle inhibition can be performed, e.g., by the subject, by a health care provider, or by a provider of cosmetics.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-2.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-1.

In a preferred embodiment, the angiogenic molecule is VEGF.

In a preferred embodiment, the wrinkle is caused by exposure to UVB radiation.

In a preferred embodiment, the agent is administered topically. In a preferred embodiment, the agent is administered sufficiently before UVB exposure, e.g., sun exposure, such that an anti-angiogenesis effect is present at the time of UVB exposure.

In a preferred embodiment, the agent is applied chronically. In a preferred embodiment, the agent is applied at least once a week, preferably 2, 3, 4, 5 times a week or daily for at least two weeks, preferably for at least 1, 2, 3, 4, 5, or 6 months. For example, the agent is applied periodically over 3-12 weeks, e.g., throughout the summer.

In a preferred embodiment, the inhibitor of angiogenesis, e.g., agent that induces an inhibitor of angiogenesis, is provided in a sterile composition.

In a preferred embodiment the method includes administering one or a more of an agent which increases TSP-2 activity, an agent which increases TSP-1 activity (TSP-1 activity can be increased by methods analogous to those described herein for increasing TSP-2 activity), or an agent which inhibits VEGF. In preferred embodiments one or more inhibitors of angiogenesis are administered.

In another aspect, the invention features a method of evaluating a test compound for the ability to induce the expression of an anti-angiogenic protein, e.g., TSP-1 or TSP-2, e.g., induce it in the skin. The method includes: providing a cell, e.g., an epidermal cell, having a transgene which includes a nucleic acid which encodes a reporter molecule functionally linked to a control region, e.g., a promoter, of an anti-angiogenesis gene, e.g., TSP-1 or TSP-2, where the reporter molecule is other than the protein encoded by the gene normally associated with the promoter; contacting the cell with a test compound; and evaluating a signal produced by the reporter molecule, the presence or strength of which is correlated with modulation of expression of the anti-angiogenesis gene by the test compound. The compound can be a protein, a polypeptide, a small molecule, e.g., a small molecule of molecular weight less than 2000 daltons, preferably less than 1000 daltons.

In a preferred embodiment, the reporter is a molecule that can provide a fluorescent signal. The reporter can be, e.g., luciferase, GFP, or BFP. In other embodiments, the reporter is an enzyme.

In a preferred embodiment, the cell is a cultured cell, e.g., an immortalized human epidermal keratinocyte.

In a preferred embodiment, the cell is from a transgenic animal.

In a preferred embodiment, the cell is from a transgenic animal and the test compound is administered to the transgenic animal, e.g., is applied topically to the skin of the transgenic animal.

In a preferred embodiment, the method further includes testing the compound *in vivo* on a human or non-human animal, e.g., by administering the compound to the animal, exposing the animal to UVB, and evaluating the effect of the compound.

In another aspect, the invention features a method of evaluating a test compound for the ability to inhibit the expression of an angiogenic protein, e.g., VEGF, e.g., inhibit it in the skin. The method includes: providing a cell, e.g., an epidermal cell, having a transgene which includes a nucleic acid which encodes a reporter molecule functionally linked to a control region, e.g., a promoter, of an angiogenic factor gene, e.g., VEGF, where the reporter molecule is other than the protein encoded by the gene normally associated with the promoter; contacting the cell with a test compound; and evaluating a signal produced by the reporter molecule, the presence or strength of which is correlated with modulation of expression of the angiogenesis gene by the test compound. The compound can be a protein, a polypeptide, a small molecule, e.g., a small molecule of molecular weight less than 2000 daltons, preferably less than 1000 daltons, more preferably less than 500 daltons.

In a preferred embodiment, the reporter is a molecule that can provide a fluorescent signal. The reporter can be, e.g., luciferase, GFP, or BFP. In other embodiments, the reporter is an enzyme.

In a preferred embodiment, the cell is a cultured cell, e.g., an immortalized human epidermal keratinocyte.

In a preferred embodiment, the cell is from a transgenic animal.

In a preferred embodiment, the cell is from a transgenic animal and the test compound is administered to the transgenic animal, e.g., is applied topically to the skin of the transgenic animal.

In a preferred embodiment, the method further includes testing the compound *in vivo* on a human or non-human animal, e.g., by administering the compound to the animal, exposing the animal to UVB, and evaluating the effect of the compound.

In another aspect, the invention features a composition for preventing or treating UVB-induced skin damage, e.g., wrinkles. The composition includes an inhibitor of angiogenesis (e.g., TSP-1 or TSP-2), e.g., an agent, e.g., a small molecule, that increases or induces TSP-1 or TSP-2; or an agent, e.g., a small molecule, that inhibits an angiogenic molecule, e.g., an agent that inhibits VEGF, and a pharmaceutically acceptable carrier. Preferably, the composition is sterile.

In a preferred embodiment, the agent is a compound, e.g., small molecule, which induces TSP-2.

In a preferred embodiment, the composition is administered topically.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-2.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-1.

In a preferred embodiment one or a more of: an agent which increases TSP-2 activity, an agent which increases TSP-1 activity, or an agent which inhibits VEGF activity are included. In preferred embodiments one or more inhibitors of angiogenesis are included.

In a preferred embodiment, the composition also includes a cosmetic ingredient, e.g., a fragrance, a humectant, or a sunscreen, e.g., octyl methoxycinnamate, aminobenzoic acid, oxybenzone, padimate O, homosalate, or titanium dioxide. The composition can also include a plant extract, e.g., aloe extract, grape extract. The composition can also include a vitamin, e.g., a vitamin A, e.g., retinol; a vitamin C, e.g., L-ascorbic acid or L-ascorbic acid palmitate; a vitamin E, e.g., tocopherol acetate.

In another aspect, the invention features a method of providing protection from UVB-induced skin damage, e.g., long term UVB-induced skin damage, e.g., wrinkle protection, to a subject. The method includes supplying to the subject a composition that includes an inhibitor of angiogenesis, e.g., an agent, e.g., a small molecule, that increases or induces an inhibitor of angiogenesis, e.g., TSP-2 or TSP-1 or an agent, e.g., a small molecule, that inhibits an angiogenic molecule, e.g., an agent that inhibits VEGF; and

supplying to the subject instructions for using the composition to prevent or reduce UVB-induced skin damage, e.g., long term UVB-induced skin damage, e.g., wrinkles.

In a preferred embodiment, the instructions include directions to apply the composition to the skin prior to and/or during sun exposure.

In a preferred embodiment, the instructions include directions to apply the composition chronically. In a preferred embodiment, the instructions include directions to apply the composition at least once a week, preferably 2, 3, 4, 5 times a week or daily for at least two weeks, preferably for at least 1, 2, 3, 4, 5, or 6 months. For example, the instructions can include instructions to apply the composition periodically over 3-12 weeks, e.g., throughout the summer.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-2.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-1.

In a preferred embodiment, the agent is a compound, e.g., small molecule, which induces TSP-2 or TSP-1.

In a preferred embodiment, the composition further comprises a cosmetic ingredient, e.g., a fragrance, or a sunscreen, e.g., octyl methoxycinnamate, aminobenzoic acid, oxybenzone, padimate O, homosalate, or titanium dioxide. The composition can also include a plant extract, e.g., aloe extract, grape extract. The composition can also include a vitamin, e.g., a vitamin A, e.g., retinol; a vitamin C, e.g., L-ascorbic acid or L-ascorbic acid palmitate; a vitamin E, e.g., tocopherol acetate.

In a preferred embodiment, the composition includes one or a more of an agent which increases TSP-2 activity, an agent which increases TSP-1 activity, or an agent which inhibits VEGF. In preferred embodiments the composition includes one or more inhibitors of angiogenesis.

In another aspect, the invention features a kit for preventing UVB-induced skin damage, e.g., long term UVB-induced skin damage, e.g., wrinkles in a subject. The kit includes a composition including an inhibitor of angiogenesis, e.g., an agent, e.g., a small

molecule, that increases or induces an inhibitor of angiogenesis; and instructions for using the composition to prevent UVB-induced skin damage, e.g., long term UVB-induced skin damage, e.g., wrinkles.

In a preferred embodiment, the agent is a compound, e.g., small molecule, which induces TSP-2.

In a preferred embodiment, the instructions include directions to apply the composition to the skin prior to and/or during sun exposure.

In a preferred embodiment, the instructions include directions to apply the composition chronically. In a preferred embodiment, the instructions include directions to apply the composition at least once a week, preferably 2, 3, 4, 5 times a week or daily for at least two weeks, preferably for at least 1, 2, 3, 4, 5, or 6 months. For example, the instructions can include instructions to apply the composition periodically over 3-12 weeks, e.g., throughout the summer.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-2.

In a preferred embodiment, the inhibitor of angiogenesis is TSP-1.

In a preferred embodiment, the composition also includes a cosmetic ingredient, e.g., a fragrance, a moisturizer, or a sunscreen, e.g., octyl methoxycinnamate, aminobenzoic acid, oxybenzone, padimate O, homosalate, or titanium dioxide. The composition can also include a plant extract, e.g., aloe extract, grape extract. The composition can also include a vitamin, e.g., a vitamin A, e.g., retinol; a vitamin C, e.g., L-ascorbic acid or L-ascorbic acid palmitate; a vitamin E, e.g., tocopherol acetate.

In a preferred embodiment, the instructions include directions to apply the composition to the skin, e.g., exposed skin, e.g., the face, neck, hands, ears, chest, or bald areas of the scalp. Preferably, the instructions include directions to apply the composition before and/or during UVB, e.g., sun, exposure.

In a preferred embodiment, the composition includes one or a more of an agent which increases TSP-2 activity, an agent which increases TSP-1 activity, or an agent which inhibits VEGF. In preferred embodiments the composition includes one or more inhibitors of angiogenesis.

A wrinkle, as used herein, is a configuration change in the surface of the skin. There may or may not be a specific structural alterations at the histological level of a wrinkle. Wrinkles can be classified as described in Kligman et al. (1985) *Br J Derm* 113:37-42, herein incorporated by reference. Kligman classifies wrinkles into three classes: linear wrinkles, glyphic wrinkles, and crinkles. Linear wrinkles are straight, found generally in the facial skin, and are caused by natural aging and exposure to ultraviolet light. Glyphic wrinkles are shaped as apparent triangles or rectangles of wrinkles, are found on the face, hands, and neck exposed to sunlight, and are aggravated by exposure to ultraviolet light or dermatoheliosis. Crinkles are thin, crinkled wrinkles on flabby skin, found anywhere on the skin, but typically on the backs of hands and around the eyelids. Wrinkles include, and are sometimes referred to as, lines, fine wrinkles, crinkles, crow's feet, or sags.

The term "small molecule", as used herein, includes peptides, peptidomimetics, or non-peptidic compounds, such as organic molecules, having a molecular weight less than 2,000, preferably less than 1,000 daltons.

Treating, as used herein, can mean total or partial alleviation or elimination of a symptom or effect of a disorder. Preventing, as used herein, can mean complete prevention, or a delay in the appearance, of a symptom or effect of a disorder.

As used herein, exposure to long-term (or chronic) UVB-radiation means chronic exposure to natural sunlight or artificial UVB radiation (e.g., a UVB sun lamp, e.g., for tanning, or for phototherapy, e.g., for treatment of psoriasis, atopic dermatitis, or vitiligo). For example, chronic exposure can be exposure to the sun at a UV index of 3-6, or higher, for at least 10 minutes at least 3, more preferably at least 5, or at least 10 times in a preselected period of time. The preselected period of time can be 1 month, 2 months, 3 months, 6 months, 12 months or 24 months, e.g., exposure to a cumulative 5 hours of UVB radiation, e.g., sunlight or artificial UVB radiation, in a 12 month period. A subject at risk of long term UV-induced damage, e.g., wrinkles, can be a subject who has been, or

will be, exposed to at least 10 minutes of sun at a UV index of 3-6, or higher, at least 10 times during a one year period, or a subject who has been or will be exposed to a cumulative 5 hours of UVB radiation in one year. Preferably, the subject is exposed to at least 30 minutes of UVB radiation at least 20 times a year for at least 3 years. Preferably, the subject is exposed to the sun between 11 A.M. and 3 P.M., or the subject is exposed to the sun during the summer months, or the subject is exposed to the sun on days of high to extreme UV index. A subject at risk for long term UVB induced skin damage, e.g., wrinkles, includes, e.g., a person who lives at a high altitude, e.g., a person who lives at least 1000 feet above sea level; a person who lives near the equator, e.g., within 1000 miles from the equator; a person who participates in outdoor sports at least 10 times in one year, e.g., a person who participates in jogging, playing tennis, mountain climbing; snow skiing, or water skiing; a person who is undergoing or has undergone UVB phototherapy.

Detailed Description

Exposure to UVB radiation

The major source of UVB radiation is natural sunlight. The intensity of UVB rays varies depending on the time of day, time of year, the sun's position in the sky, altitude and distance from the equator. These rays are most intense during the midday hours in the summer, although they are always present, even during the winter months. Distance above sea level and distance from the equator are also important to consider. The higher the altitude the greater the intensity of UVB rays. Therefore, mountaineers, skiers, and those who live at high altitudes are at risk of long term UVB damage. Also, the nearer one is to the equator the more intense the UV radiation and the higher the risk of long term UVB damage.

Snow, water, and sand reflect sunlight, magnifying the amount of UVB radiation that reaches the skin. Even when clouds obscure the sun, UVB levels can still be sufficiently high to cause photoaging, e.g., wrinkles, upon long term exposure.

The UV index (developed by the Environmental Protection Agency) indicates the intensity of the sun's UV rays on a given day. There are four categories – moderate (UV index is less than 3), high (UV index is 3 to 6) very high (UV index is 6 to 10) and extreme (UV index is greater than 10). A moderate UV Index means it will take more than an hour to burn your skin; an extreme level means it will take less than 15 minutes. The index is often included with weather reports. Clinically, UVB exposure is measured in MEDs. One MED is the amount of UVB required to produce a sunburn in sensitive skin. Because the effects of UVB exposure are cumulative, long term or chronic UVB induced wrinkles can occur as a result of long term exposure to UVB levels below those which, upon acute exposure, can cause erythema or edema or burning (e.g., below one MED). For example, a subject is at risk of long term UVB induced wrinkles if the subject is chronically exposed to the sun even if the subject is only exposed to the sun during days with a low or moderate UV Index.

Angiogenesis and chronic UVB exposure

Photoaged skin is characterized by epidermal hyperplasia, dermal elastosis and matrix protein degradation (5, 38), and by the presence of a perivenular lymphohistocytic dermal infiltrates (23). Results described herein reveal that chronic UVB irradiation of the skin is associated with pronounced cutaneous angiogenesis and with increased VEGF expression in the hyperplastic epidermis, and that targeted inhibition of skin angiogenesis by TSP-1 prevents UVB-induced dermal damage and wrinkle formation.

After 10 weeks of UVB irradiation of Skh-1 hairless mice, an established experimental model for chronic photoaging (26), we found pronounced wrinkle formation and the characteristic histological features of epidermal and dermal hyperplasia, associated with increased detection of disorganized elastic and collagen fibers in the dermis. Computer-assisted quantitative image analysis (24) of tissue sections stained for the endothelial junction molecule CD31 (39) revealed a marked induction of skin angiogenesis after long-term UVB irradiation, with a significant increase of both vessel density and vessel size. These vascular changes were comparable to the angiogenic

changes which occur during cutaneous wound healing where both sprouting of preexisting blood vessels and vessel enlargement contribute to the formation of the vessel-rich granulation tissue (24). In contrast, chronic inflammatory skin diseases such as psoriasis predominantly show vascular remodeling with elongation and enlargement of cutaneous microvessels but without the formation of new vessel sprouts. These findings indicate that chronic UVB irradiation of the skin results in a chronic tissue repair reaction and they suggest that angiogenesis might play an important role in the mediation of UVB-induced skin damage.

Vascular endothelial growth factor (VEGF) has been identified as a major, keratinocyte-derived skin angiogenesis factor (40) with increased expression in the hyperplastic epidermis of lesional psoriatic skin (12) and of other skin diseases associated with dermal angiogenesis (14, 41), as well as in the neo-epidermis of healing wounds (13, 42). In the experiments described herein, a pronounced upregulation of VEGF mRNA expression was found in the hyperplastic epidermis of chronically UVB-irradiated skin, preferentially in suprabasal keratinocytes. These findings are in accordance with previous reports that acute UVB irradiation induced VEGF expression in human epidermal keratinocyte in vitro (43) (44) and in vivo (7).

Angiogenesis and Chronic UVB-Induced Wrinkles

Transgenic mice with skin-specific overexpression of the angiogenesis inhibitor TSP-1 were exposed to chronic UVB irradiation. Using an established keratin 14 (K14) promoter cassette to target TSP-1 transgene expression to epidermal keratinocytes, we have previously established K14/TSP-1 transgenic mice which are characterized by increased levels of epidermal TSP-1 expression, by normal thickness and morphology of the epidermis and dermis and by potent inhibition of skin angiogenesis during cutaneous wound healing (24). The use of the K14 promoter ensures high transgene expression under conditions of epidermal hyperplasia because K14 gene expression is greatly enhanced in proliferating keratinocytes. Results described in the Examples herein revealed that epidermal overexpression of TSP-1 potentially inhibited dermal photodamage

and collagen and elastic fiber disorganization, and also completely inhibited the formation of skin wrinkles. This was associated with a potent inhibition of skin angiogenesis and with decreased endothelial proliferation rates and with increased apoptosis of endothelial cells. Together, these results indicate that inhibition of the repair-associated, UVB-induced angiogenesis also prevents dermal photodamage including the formation of wrinkles.

It has been previously shown that TSP-1 mediates inhibition of angiogenesis by specific interactions of distinct sequences within the type I repeats with the CD36 receptor on endothelial cells, resulting in enhanced endothelial cell apoptosis rates (46). Recent evidence suggests that TSP-1, similar to the related molecule TSP-2 (47), can also inhibit the activation of matrix metalloproteinase-2 (MMP-2), with important implications for its antiangiogenic effects (48, 49). These results identify an additional mechanism by which TSP-1, through inhibition of MMP-9 activation, may reduce cutaneous angiogenesis induced by UVB irradiation. MMP-9 is a member of a zinc proteinase family of molecules that digest components of the extracellular matrix, and increased levels of MMP-9 expression and activity have been found in UV irradiated human skin (35, 50, 51).

Conversely, TSP-2 knock out mice showed increased wrinkling in response to long term UVB exposure, as compared to wildtype mice. However, no major differences in MMP-9 activity were detected between TSP-2 knock out mice and wildtype mice after chronic UVB irradiation. These results suggest that the specific inhibition of skin angiogenesis (as opposed to MMP effects) may represent a promising new approach for the prevention of chronic UVB damage, e.g., wrinkles, to the skin.

Analogues of TSP

Analogues can differ from naturally occurring TSP-1 or TSP-2 in amino acid sequence or in ways that do not involve sequence, or both. Non-sequence modifications include *in vivo* or *in vitro* chemical derivatization of TSP-1 or TSP-2. Non-sequence

modifications include changes in acetylation, methylation, phosphorylation, carboxylation, or glycosylation.

The sequence of TSP-1 and TSP-2, e.g., human TSP-1 and TSP-2, are known in the art. Preferred analogs include TSP-1 or TSP-2 (or biologically active fragments thereof) whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the TSP-1 or TSP-2 biological activity. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other conservative substitutions can be taken from the table below.

TABLE 1
CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D- homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β -Ala Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met

Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thiazolidine-4- carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Other analogs within the invention are those with modifications which increase peptide stability; such analogs may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids; and cyclic analogs.

Production of Fragments and Analogs

Generation of Fragments

Fragments of a protein can be produced in several ways, e.g., recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide

fragments. Digestion with "end-nibbling" endonucleases can thus generate DNA's which encode an array of fragments. DNA's which encode fragments of a protein can also be generated by random shearing, restriction digestion or a combination of the above-discussed methods.

Fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, peptides of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Random Methods

Amino acid sequence variants of a protein can be prepared by random mutagenesis of DNA which encodes a protein or a particular domain or region of a protein. Useful methods include PCR mutagenesis and saturation mutagenesis. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences. (Methods for screening proteins in a library of variants are elsewhere herein.)

PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity is used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized is amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments are inserted into appropriate cloning vectors to provide random mutant libraries.

Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments both neutral substitutions, as well as those that alter function, are obtained. The distribution of point mutations is not biased toward conserved sequence elements.

Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of a degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Directed Mutagenesis

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create

variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989). In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions are then refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983). Briefly, the desired DNA is altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the

desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci.* (1978) USA, 75: 5765).

Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene* (1985) 34:315). The starting material is a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. For example, the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

Primary High-Through-Put Methods for Screening Libraries of Peptide Fragments or Homologs

Various techniques are known in the art for screening generated mutant gene products. Techniques for screening large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, assembly into a trimeric molecules, binding to natural ligands, e.g., a receptor or substrates, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

Two Hybrid Systems

Two hybrid (interaction trap) assays can be used to identify a protein that interacts with VEGF. These may include agonists, superagonists, and antagonists. (The subject protein and a protein it interacts with are used as the bait protein and fish proteins.). These assays rely on detecting the reconstitution of a functional transcriptional activator mediated by protein-protein interactions with a bait protein. In particular, these assays

make use of chimeric genes which express hybrid proteins. The first hybrid comprises a DNA-binding domain fused to the bait protein. e.g., a TSP-1 or TSP-2 molecule or a fragment thereof. The second hybrid protein contains a transcriptional activation domain fused to a "fish" protein, e.g. an expression library. If the fish and bait proteins are able to interact, they bring into close proximity the DNA-binding and transcriptional activator domains. This proximity is sufficient to cause transcription of a reporter gene which is operably linked to a transcriptional regulatory site which is recognized by the DNA binding domain, and expression of the marker gene can be detected and used to score for the interaction of the bait protein with another protein.

Display Libraries

In one approach to screening assays, the candidate peptides are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to bind an appropriate receptor protein via the displayed product is detected in a "panning assay". For example, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, a detectably labeled ligand can be used to score for potentially functional peptide homologs. Fluorescently labeled ligands, e.g., receptors, can be used to detect homolog which retain ligand-binding activity. The use of fluorescently labeled ligands, allows cells to be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, to be separated by a fluorescence-activated cell sorter.

A gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at concentrations well over 10^{13} phage per milliliter, a large number of phage can be screened at one time. Second, since each infectious phage displays a gene product on its surface, if a particular phage is

recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd., and f1 are most often used in phage display libraries. Either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle. Foreign epitopes can be expressed at the NH₂-terminal end of pIII and phage bearing such epitopes recovered from a large excess of phage lacking this epitope (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

A common approach uses the maltose receptor of *E. coli* (the outer membrane protein, LamB) as a peptide fusion partner (Charbit et al. (1986) *EMBO* 5, 3029-3037). Oligonucleotides have been inserted into plasmids encoding the LamB gene to produce peptides fused into one of the extracellular loops of the protein. These peptides are available for binding to ligands, e.g., to antibodies, and can elicit an immune response when the cells are administered to animals. Other cell surface proteins, e.g., OmpA (Schorr et al. (1991) *Vaccines* 91, pp. 387-392), PhoE (Agterberg, et al. (1990) *Gene* 88, 37-45), and PAL (Fuchs et al. (1991) *Bio/Tech* 9, 1369-1372), as well as large bacterial surface structures have served as vehicles for peptide display. Peptides can be fused to pilin, a protein which polymerizes to form the pilus-a conduit for interbacterial exchange of genetic information (Thiry et al. (1989) *Appl. Environ. Microbiol.* 55, 984-993). Because of its role in interacting with other cells, the pilus provides a useful support for the presentation of peptides to the extracellular environment. Another large surface structure used for peptide display is the bacterial motive organ, the flagellum. Fusion of peptides to the subunit protein flagellin offers a dense array of many peptides copies on the host cells (Kuwayama et al. (1988) *Bio/Tech.* 6, 1080-1083). Surface proteins of other bacterial species have also served as peptide fusion partners. Examples include the *Staphylococcus* protein A and the outer membrane protease IgA of *Neisseria* (Hansson et al. (1992) *J. Bacteriol.* 174, 4239-4245 and Klauser et al. (1990) *EMBO J.* 9, 1991-1999).

In the filamentous phage systems and the LamB system described above, the physical link between the peptide and its encoding DNA occurs by the containment of the DNA within a particle (cell or phage) that carries the peptide on its surface. Capturing the peptide captures the particle and the DNA within. An alternative scheme uses the DNA-binding protein LacI to form a link between peptide and DNA (Cull *et al.* (1992) *PNAS USA* 89:1865-1869). This system uses a plasmid containing the LacI gene with an oligonucleotide cloning site at its 3'-end. Under the controlled induction by arabinose, a LacI-peptide fusion protein is produced. This fusion retains the natural ability of LacI to bind to a short DNA sequence known as LacO operator (LacO). By installing two copies of LacO on the expression plasmid, the LacI-peptide fusion binds tightly to the plasmid that encoded it. Because the plasmids in each cell contain only a single oligonucleotide sequence and each cell expresses only a single peptide sequence, the peptides become specifically and stably associated with the DNA sequence that directed its synthesis. The cells of the library are gently lysed and the peptide-DNA complexes are exposed to a matrix of immobilized receptor to recover the complexes containing active peptides. The associated plasmid DNA is then reintroduced into cells for amplification and DNA sequencing to determine the identity of the peptide ligands. As a demonstration of the practical utility of the method, a large random library of dodecapeptides was made and selected on a monoclonal antibody raised against the opioid peptide dynorphin B. A cohort of peptides was recovered, all related by a consensus sequence corresponding to a six-residue portion of dynorphin B. (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89-1869)

This scheme, sometimes referred to as peptides-on-plasmids, differs in two important ways from the phage display methods. First, the peptides are attached to the C-terminus of the fusion protein, resulting in the display of the library members as peptides having free carboxy termini. Both of the filamentous phage coat proteins, pIII and pVIII, are anchored to the phage through their C-termini, and the guest peptides are placed into the outward-extending N-terminal domains. In some designs, the phage-displayed peptides are presented right at the amino terminus of the fusion protein. (Cwirla, *et al.*

(1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 6378-6382) A second difference is the set of biological biases affecting the population of peptides actually present in the libraries. The LacI fusion molecules are confined to the cytoplasm of the host cells. The phage coat fusions are exposed briefly to the cytoplasm during translation but are rapidly secreted through the inner membrane into the periplasmic compartment, remaining anchored in the membrane by their C-terminal hydrophobic domains, with the N-termini, containing the peptides, protruding into the periplasm while awaiting assembly into phage particles. The peptides in the LacI and phage libraries may differ significantly as a result of their exposure to different proteolytic activities. The phage coat proteins require transport across the inner membrane and signal peptidase processing as a prelude to incorporation into phage. Certain peptides exert a deleterious effect on these processes and are underrepresented in the libraries (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251). These particular biases are not a factor in the LacI display system.

The number of small peptides available in recombinant random libraries is enormous. Libraries of 10^7 - 10^9 independent clones are routinely prepared. Libraries as large as 10^{11} recombinants have been created, but this size approaches the practical limit for clone libraries. This limitation in library size occurs at the step of transforming the DNA containing randomized segments into the host bacterial cells. To circumvent this limitation, an *in vitro* system based on the display of nascent peptides in polysome complexes has recently been developed. This display library method has the potential of producing libraries 3-6 orders of magnitude larger than the currently available phage/phagemid or plasmid libraries. Furthermore, the construction of the libraries, expression of the peptides, and screening, is done in an entirely cell-free format.

In one application of this method (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251), a molecular DNA library encoding 10^{12} decapeptides was constructed and the library expressed in an *E. coli* S30 *in vitro* coupled transcription/translation system. Conditions were chosen to stall the ribosomes on the mRNA, causing the accumulation of a substantial proportion of the RNA in polysomes and yielding complexes containing nascent peptides still linked to their encoding RNA. The polysomes are sufficiently

robust to be affinity purified on immobilized receptors in much the same way as the more conventional recombinant peptide display libraries are screened. RNA from the bound complexes is recovered, converted to cDNA, and amplified by PCR to produce a template for the next round of synthesis and screening. The polysome display method can be coupled to the phage display system. Following several rounds of screening, cDNA from the enriched pool of polysomes was cloned into a phagemid vector. This vector serves as both a peptide expression vector, displaying peptides fused to the coat proteins, and as a DNA sequencing vector for peptide identification. By expressing the polysome-derived peptides on phage, one can either continue the affinity selection procedure in this format or assay the peptides on individual clones for binding activity in a phage ELISA, or for binding specificity in a completion phage ELISA (Barret, et al. (1992) *Anal. Biochem* 204,357-364). To identify the sequences of the active peptides one sequences the DNA produced by the phagemid host.

Secondary Screens

The high through-put assays described above can be followed by secondary screens in order to identify further biological activities which will, e.g., allow one skilled in the art to differentiate agonists from antagonists. The type of a secondary screen used will depend on the desired activity that needs to be tested. For example, an assay can be developed in which the ability to inhibit an interaction between a protein of interest, e.g., TSP 1 or TSP2, and a ligand can be used to identify agonists or antagonists from a group of peptide fragments isolated though one of the primary screens described above. For example, the ability of a test compound to inhibit angiogenesis in the skin can be tested by a number of methods known in the art, e.g., by applying a test compound or treatment to the skin of a subject e.g., an experimental animal (e.g., a mouse); and evaluating the number and/or size of blood vessels in the skin of the subject in the absence compared to in the presence of the compound. A compound that causes a decrease in the number or size of blood vessels in the skin of the subject is identified as a compound that inhibits angiogenesis in the skin.

Therefore, methods for generating fragments and analogs and testing them for activity are known in the art. Once the core sequence of interest is identified, it is routine for one skilled in the art to obtain analogs and fragments and to test them for the desired activity.

Peptide Mimetics

The invention also provides for reduction of the protein binding domains of the subject TSP-1 or TSP-2 polypeptides to generate mimetics, e.g. peptide or non-peptide agents. See, for example, "Peptide inhibitors of human papillomavirus protein binding to retinoblastoma gene protein" European patent applications EP 0 412 762 and EP 0 031 080.

Non-hydrolyzable peptide analogs of critical residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Fusion Proteins

Polypeptides for modulating the level of TSP-1 or TSP-2 protein can be fused to another protein or portion thereof. For example, a TSP-1 or TSP-2 protein or portion thereof, can be operably linked to another polypeptide moiety to enhance solubility. Examples of a protein which can be fused with TSP-1 or TSP-2 or portions thereof

include a plasma protein or fragment thereof, which can improve the circulating half life of VEGF. For example, the fusion protein can be a TSP-1 or TSP-2 -immunoglobulin (Ig) fusion protein in which the TSP-1 or TSP-2 sequence is fused to a sequence derived from the immunoglobulin superfamily. Several soluble fusion protein constructs have been disclosed wherein the extracellular domain of a cell surface glycoprotein is fused with the constant F(c) region of an immunoglobulin. For example, Capon et al. (1989) *Nature* 337(9):525-531, provide guidance on generating a longer lasting CD4 analog by fusing CD4 to an immunoglobulin (IgG1). *See also*, Capon et al., U.S. Patent Numbers: 5,116,964 and 5,428,130 (CD4-IgG fusion constructs); Linsley et al., U.S. Patent Number 5,434,131 (CTLA4-IgG1 and B7-IgG1 fusion constructs); Linsley et al. (1991) *J. Exp. Med.* 174:561-569 (CTLA4-IgG1 fusion constructs); and Linsley et al. (1991) *J. Exp. Med.* 173:721-730 (CD28-IgG1 and B7-IgG1 fusion constructs). Such fusion proteins have proven useful for modulating receptor-ligand interactions and reducing inflammation *in vivo*. For example, fusion proteins in which an extracellular domain of cell surface tumor necrosis factor receptor (TNFR) proteins has been fused to an immunoglobulin constant (Fc) region have been used *in vivo*. *See, for example*, Moreland et al. (1997) *N. Engl. J. Med.* 337(3):141-147; and, van der Poll et al. (1997) *Blood* 89(10):3727-3734).

Antisense Nucleic Acid Sequences

Nucleic acid molecules which are antisense to a nucleotide encoding a positive angiogenesis factor, e.g., VEGF, can be used as an agent which inhibits angiogenesis in the methods described herein. An "antisense" nucleic acid includes a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a positive angiogenesis factor, e.g., VEGF, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can form hydrogen bonds with a sense nucleic acid. The antisense nucleic acid can be complementary to an entire VEGF coding strand, or to only a portion

thereof. For example, an antisense nucleic acid molecule which antisense to the "coding region" of the coding strand of a nucleotide sequence encoding VEGF can be used.

The coding strand sequences encoding VEGF are known. Given the coding strand sequences encoding VEGF, antisense nucleic acids can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of VEGF mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of VEGF mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of VEGF mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-

carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest.

RNAi

Double stranded nucleic acid molecules that can silence a gene encoding a component of the IR signaling pathway described herein, e.g., a component described herein, can also be used as an agent which inhibits expression of the component of the IR signaling pathway. RNA interference (RNAi) is a mechanism of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) corresponding to a gene (or coding region) of interest is introduced into a cell or an organism, resulting in degradation of the corresponding mRNA. The RNAi effect persists for multiple cell divisions before gene expression is regained. RNAi is therefore an extremely powerful method for making targeted knockouts or "knockdowns" at the RNA level. RNAi has proven successful in human cells, including human embryonic kidney and HeLa cells (see, e.g., Elbashir et al. *Nature* 2001 May 24;411(6836):494-8). In one embodiment, gene silencing can be induced in mammalian cells by enforcing endogenous expression of RNA hairpins (see Paddison et al., 2002, *PNAS USA* 99:1443-1448). In another embodiment, transfection of small (21-23 nt) dsRNA specifically inhibits gene expression (reviewed in Caplen (2002) *Trends in Biotechnology* 20:49-51)..

Briefly, RNAi is thought to work as follows. dsRNA corresponding to a portion of a gene to be silenced is introduced into a cell. The dsRNA is digested into 21-23 nucleotide siRNAs, or short interfering RNAs. The siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or RISC. The RISC targets the homologous transcript by base pairing interactions between one of the siRNA strands and the endogenous mRNA. It then cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA (reviewed in Sharp et al (2001) *Genes Dev* 15: 485-490; and Hammond et al. (2001) *Nature Rev Gen* 2: 110-119).

RNAi technology in gene silencing utilizes standard molecular biology methods. dsRNA corresponding to the sequence from a target gene to be inactivated can be produced by standard methods, e.g., by simultaneous transcription of both strands of a template DNA (corresponding to the target sequence) with T7 RNA polymerase. Kits for production of dsRNA for use in RNAi are available commercially, e.g., from New England Biolabs, Inc. Methods of transfection of dsRNA or plasmids engineered to make dsRNA are routine in the art.

Gene silencing effects similar to those of RNAi have been reported in mammalian cells with transfection of a mRNA-cDNA hybrid construct (Lin et al., *Biochem Biophys Res Commun* 2001 Mar 2;281(3):639-44), providing yet another strategy for gene silencing.

Administration

An agent which modulates angiogenesis, e.g., an angiogenesis inhibitor, e.g., TSP-1 or TSP-2, or an agent which modulates TSP-21 or TSP-2, can be administered to a subject by standard methods. For example, the agent can be administered by any of a number of different routes including intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), and transmucosal. In one embodiment, the TSP-1 or TSP-2 or modulating agents thereof can be administered topically.

The agent which modulates TSP-1 or TSP-2 protein levels, e.g., TSP-1 or TSP-2 nucleic acid molecules, TSP-1 or TSP-2 polypeptides, fragments or analogs, TSP-1 or TSP-2 modulators, and anti- TSP-1 or TSP-2 antibodies (also referred to herein as "active compounds") can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. Such compositions typically include the nucleic acid molecule, polypeptide, modulator, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically

active substances are known. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition can be formulated to be compatible with its intended route of administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and

antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a TSP-1 or TSP-2 polypeptide) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art. Such transdermal formulations can be applied to the skin to promote or inhibit hair growth.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

The nucleic acid molecules described herein can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al., *PNAS* 91:3054-3057, 1994). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

The agent which modulates the level of angiogenesis, e.g., TSP-1 or TSP-2 polypeptide or fragment or analog thereof, can be administered by locally administration, e.g., topical administration. The agent can be applied once or it can be administered continuously, e.g., the agent is administered with sufficient frequency such that the effect on the TSP-1 or TSP-2 protein level is maintained for a selected period, e.g., 5, 10, 20, 30, 50, 90, 180, 365 days or more. The administration of an agent which modulates, e.g., increases or inhibits, the level of a TSP-1 or TSP-2 protein, e.g., a TSP-1 or TSP-2 polypeptide or fragment or analog thereof, can also be repeated.

Gene Therapy

Gene constructs of the invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either a positive or negative angiogenesis factor, e.g., an angiogenesis inhibitor, e.g., a TSP-1 or TSP-2 polypeptide or fragment or analog thereof. The invention features expression vectors for *in vivo* transfection and expression of a TSP-1 or TSP-2 polypeptide in particular cell types, e.g., epidermal cells, so as to inhibit angiogenesis, e.g., in the epidermis. Expression constructs of TSP-1 or TSP-2 polypeptides, may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the TSP-1 or TSP-2 gene to cells *in vivo*. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO₄ precipitation carried out *in vivo*.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding a TSP-1 or TSP-2 polypeptide, or a VEGF antisense nucleic acid. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors can be used as a recombinant gene delivery system for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A replication defective retrovirus can be packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA*

88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld et al. (1992) cited *supra*). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis *in situ* where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267).

Yet another viral vector system useful for delivery of the subject gene is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al.

(1992) *Curr. Topics in Micro. and Immunol.* 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a TSP-1 or TSP-2 polypeptide, fragment, or analog, in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject TSP-1 or TSP-2 gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes. Other embodiments include plasmid injection systems such as are described in Meuli et al. (2001) *J Invest Dermatol.* 116(1):131-135; Cohen et al. (2000) *Gene Ther* 7(22):1896-905; or Tam et al. (2000) *Gene Ther* 7(21):1867-74.

In a representative embodiment, a gene encoding a TSP-1 or TSP-2 polypeptide, active fragment, or analog, can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075).

In clinical settings, the gene delivery systems for the therapeutic TSP-1 or TSP-2 gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced in tact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

Cell Therapy

TSP-1 or TSP-2 can also be increased in a subject by introducing into a cell, e.g., an epidermal cell, e.g., a keratinocyte, a nucleotide sequence that modulates the production of TSP-1 or TSP-2, e.g., a nucleotide sequence encoding a TSP-1 or TSP-2 polypeptide or functional fragment or analog thereof, a promoter sequence, e.g., a promoter sequence from a TSP-1 or TSP-2 gene or from another gene; an enhancer sequence, e.g., 5' untranslated region (UTR), e.g., a 5' UTR from a TSP-1 or TSP-2 gene or from another gene, a 3' UTR, e.g., a 3' UTR from a TSP-1 or TSP-2 gene or from another gene; a polyadenylation site; an insulator sequence; or another sequence that modulates the expression of TSP-1 or TSP-2. The cell can then be introduced into the subject.

Primary and secondary cells to be genetically engineered can be obtained from a variety of tissues and include cell types which can be maintained propagated in culture. For example, primary and secondary cells include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells (myoblasts) and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the genetically engineered primary or secondary cells are administered. However, primary cells may be obtained for a donor (other than the recipient) of the same species or another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse).

The term "primary cell" includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term "secondary cell" or "cell strain" refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to herein as a secondary cell, as are all cells in subsequent passages. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times. A cell strain consists of secondary cells that: 1) have been passaged one or more times; 2) exhibit a finite number of mean population doublings in culture; 3) exhibit the properties of contact-inhibited, anchorage dependent growth (anchorage-dependence does not apply to cells that are propagated in suspension culture); and 4) are not immortalized. A "clonal cell strain" is defined as a cell strain that is derived from a single founder cell. A "heterogenous cell strain" is defined as a cell strain that is derived from two or more founder cells.

Primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected with an exogenous nucleic acid sequence which includes a nucleic acid sequence encoding a signal peptide, and/or a heterologous nucleic acid sequence, e.g., encoding TSP-1 or TSP-2, and produce the encoded product stably and reproducibly *in*

vitro and *in vivo*, over extended periods of time. A heterologous amino acid can also be a regulatory sequence, e.g., a promoter, which causes expression, e.g., inducible expression or upregulation, of an endogenous TSP-1 or TSP-2 sequence. An exogenous nucleic acid sequence can be introduced into a primary or secondary cell by homologous recombination as described, for example, in U.S. Patent No.: 5,641,670, the contents of which are incorporated herein by reference.

The transfected primary or secondary cells may also include DNA encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation. Methods for producing transfected primary and secondary cells which stably express exogenous synthetic DNA, clonal cell strains and heterogeneous cell strains of such transfected cells, methods of producing the clonal heterogeneous cell strains, and methods of treating or preventing an abnormal or undesirable condition through the use of populations of transfected primary or secondary cells are part of the present invention.

Transfection of Primary or Secondary Cells of Clonal or Heterogeneous Cell Strains

Vertebrate tissue can be obtained by standard methods such a punch biopsy or other surgical methods of obtaining a tissue source of the primary cell type of interest. For example, punch biopsy is used to obtain skin as a source of fibroblasts or keratinocytes. A mixture of primary cells is obtained from the tissue, using known methods, such as enzymatic digestion or explanting. If enzymatic digestion is used, enzymes such as collagenase, hyaluronidase, dispase, pronase, trypsin, elastase and chymotrypsin can be used.

The resulting primary cell mixture can be transfected directly or it can be cultured first, removed from the culture plate and resuspended before transfection is carried out. Primary cells or secondary cells are combined with exogenous nucleic acid sequence to, e.g., stably integrate into their genomes, and treated in order to accomplish transfection. The exogenous nucleic acid sequence can optionally include DNA encoding a selectable marker. The exogenous nucleic acid sequence and selectable marker-encoding DNA can

either be on separate constructs or on a single construct. An appropriate quantity of DNA is used to ensure that at least one stably transfected cell containing and appropriately expressing exogenous DNA is produced. In general, approximately 0.1 to 500µg of DNA is used.

As used herein, the term "transfection" includes a variety of techniques for introducing an exogenous nucleic acid into a cell including calcium phosphate or calcium chloride precipitation, microinjection, DEAE-dextrin-mediated transfection, lipofection or electrophoration.

Electroporation is carried out at approximate voltage and capacitance (and corresponding time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be carried out over a wide range of voltages (e.g., 50 to 2000 volts) and corresponding capacitance. Total DNA of approximately 0.1 to 500µg is generally used.

Methods such as calcium phosphate precipitation, modified calcium phosphate precipitation an polybrene precipitation, liposome fusion and receptor-mediated gene delivery can also be used to transect cells. Primary or secondary cells can also be transfected using microinjection. A stably, transfected cell can then be isolated and cultured and sub cultivated, under culturing conditions and for sufficient time to propagate stably transfected secondary cells an produce a clonal cell strain of transfected secondary cells. Alternatively, more than one transfected cell is cultured and sub cultured, resulting in production of a heterogeneous cell strain.

Transfected primary or secondary cells undergo sufficient number doubling to produce either a clonal cell strain or a heterogeneous cell strain of sufficient size to provide the therapeutic protein to an individual in effective amounts. In general, for example, 0.1cm² of skin is biopsies and assumed to contain 1,000,000 cells; one cell is used to produce a clonal cell strain and undergoes approximately 27 doublings to produce 100 million transfected secondary cells. If a heterogeneous cell strain is to be produced from an original transfected population of approximately 1000,000 cells, only 10 doublings are needed to produce 100 million transfected cells.

The number of required cells in a transfected clonal heterogeneous cell strain is variable and depends on a variety of factors, including but not limited to, the use of the transfected cells, the functional level of the exogenous DNA in the transfected cells, the site of implantation of the transfected cells (for example, the number of cells that can be used is limited by the anatomical site of implantation), and the age, surface area, and clinical condition of the patient. The put these factors in perspective, to deliver therapeutic levels of human growth hormone in an otherwise healthy 10 kg patient with isolated growth hormone deficiency, approximately one to five hundred million transfected fibroblast would be necessary (the volume of these cells is about that of the very tip of the patient's thumb).

Implantation of Clonal Cell Strain or Heterogeneous Cell Strain of Transfected Secondary Cells

The transfected cells, e.g., cells produced as described herein, can be introduced into an individual to whom the product is to be delivered. The clonal cell strain or heterogeneous cell strain is then introduced into an individual. Various routed of administration and various sites (e.g., renal sub capsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchic, intraperitoneal (including intraomental), intramuscularly implantation) can be used. One implanted in individual, the transfected cells produce the product encoded by the heterologous DNA or are affected by the heterologous DNA itself. For example, an individual who suffers from hair loss is a candidate for implantation of TSP-1 or TSP-2 producing cells.

The individual can have a small skin biopsy performed; this is a simple procedure which can be performed on an outpatient basis. The piece of skin is taken, for example, from under the arm and can require about one minute to remove. The sample is processed, resulting in isolation of the patient's cell (e.g., fibroblasts) and genetically engineered to produce TSP-1 or TSP-2 or another protein or molecule that induces the production of TSP-1 or TSP-2. Based on the age, weight, and clinical condition of the patient, the required number of cells are grown in large-scale culture. The entire process should require 4-6 weeks and, at the end of that time, the appropriate number of

genetically engineered cells are introduced into the individual, once again as an outpatient (e.g., by injecting them back under the patient's skin, e.g., on the scalp or face). The patient is now capable of producing TSP-1 or TSP-2 which can prevent or reduce wrinkles.

For some, this will be a one-time treatment and, for others, multiple cell therapy treatments will be required.

As this example suggests, the cells used will generally be patient-specific genetically engineered cells. It is possible, however, to obtain cells from another individual of the same species or from a different species. Use of such cells might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells.

Transfected primary or secondary cells can be administered alone or in conjunction with a barrier or agent for inhibiting immune response against the cell in a recipient subject. For example, an immunosuppressive agent can be administered to a subject to inhibit or interfere with normal response in the subject. Preferably, the immunosuppressive agent is an immunosuppressive drug which inhibits T cell/or B cell activity in a subject. Examples of such immunosuppressive drugs commercially available (e.g., cyclosporin A is commercially available for Sandoz Corp. East Hanover, NJ).

An immunosuppressive agent e.g., drug, can be administered to a subject at a dosage sufficient to achieve the desired therapeutic effect (e.g., inhibition of rejection of the cells). Dosage ranges for immunosuppressive drugs are known in the art. *See, e.g.,* Freed et al. (1992) *N. Engl. J. Med.* 327:1549; Spencer et al. (1992) *N. Engl. J. Med.* 327:1541; Widner et al. (1992) *n. Engl. J. Med.* 327:1556). Dosage values may vary according to factors such as the disease state, age, sex, and weight of the individual.

Another agent which can be used to inhibit T cell activity in a subject is an antibody, or fragment or derivative thereof. Antibodies capable of depleting or sequestering T cells *in vivo* are known in the art. Polyclonal antisera can be used, for example, anti-lymphocyte serum. Alternatively, one or more monoclonal antibodies can be used. Preferred T cell depleting antibodies include monoclonal antibodies which bind

to CD2, CD3, CD4, CD8, CD40, CD40, ligand on the cell surface. Such antibodies are known in the art and are commercially available, for example, from American Type Culture Collection. A preferred antibody for binding CD3 on human T cells is OKT3 (ATCC CRL 8001).

An antibody which depletes, sequesters or inhibits T cells within a recipient subject can be administered in a dose for an appropriate time to inhibit rejection of cells upon transplantation. Antibodies are preferably administered intravenously in a pharmaceutically acceptable carrier of diluent (e.g., saline solution).

Another way of interfering with or inhibiting an immune response to the cells in a recipient subject is to use an immunobarrier. An "immunobarrier" as used herein, refers to a device which serves as a barrier between the administered cell and cells involved in immune response in a subject. For example, the cells can be administered in an implantable device. An implantable device can include the cells contained within a semi-permeable barrier, i.e., one which lets nutrients and the product diffuse in and out of the barrier but which prevents entry or larger immune system components, e.g., antibodies or complement. An implantable device typically includes a matrix, e.g., a hydrogel, biocompatible mesh, or core in which cells are disposed. Optionally, a semi permeable coating can enclose the gel. If disposed within the gel core, the administered cells should be sequestered from the cells of the immune system and should be cloaked from the cells and cytotoxic antibodies of the host. Preferably, a permselective coating such as PLL or PLO is used. The coating often has a porosity which prevents components of the recipient's immune system from entering and destroying the cells within the implantable device.

Many methods for encapsulating cells are known in the art. For example, encapsulation using a water soluble gum to obtain a semi-permeable water insoluble gel to encapsulate cells for production and other methods of encapsulation are disclosed in U.S. patent No: 4,352,883. Other implantable devices which can be used are disclosed in U.S. Patent No.: 5,084,350, U.S. Patent No. 5,427,935, WO 95/19743 published July 27,

1995, U.S. Patent No.: 5,545,423, U.S. Patent Number 4,409,331, U.S. Patent Number 4,663,286, and European Patent No. 301,777.

An advantage of the use of transfected or secondary cells is that by controlling the number of cells introduced into an individual, one can control the amount of the protein delivered to the body. In addition, in some cases, it is possible to remove the transfected cells if there is no longer a need for the product. A further advantage of treatment by use of transfected primary or secondary cells of the present invention is that production of the therapeutic product can be regulated, such as through the administration of zinc, steroids or an agent which affects transcription of a protein, product or nucleic acid product or affects the stability of a nucleic acid product.

EXAMPLES

Example 1: Enhanced skin angiogenesis after long-term UVB irradiation.

After ten weeks of UVB irradiation (cumulative dose: 5.65 J/cm²), replicas were obtained from the back skin of UVB irradiated and of non-irradiated mice in order to evaluate the skin surface relief as a parameter for the extent of skin damage. Pronounced formation of wrinkles was observed in UVB irradiated mice, whereas no visible wrinkles were detected in non-irradiated control mice. Macroscopic examination of the underside of the skin demonstrated increased subcutaneous vascularization in UVB-irradiated mice with enlarged blood vessels and increased vessel branching.

Histological analysis showed thickening of the epidermis, dermis and sebaceous glands (36) in UVB-treated mice, accompanied by accumulation of inflammatory cells in the upper dermis. Moreover, we found fragmented and less organized collagen fibers and elastic fibers in UVB irradiated skin, as compared with the regular pattern observed in non-irradiated control skin. Immunostains for CD31 revealed an increased number of enlarged blood vessels in the dermis of UVB irradiated mice, as compared with untreated

controls. These changes were most prominent in the papillary dermis, in an area immediately underlying the epidermis. Differential immunofluorescent stainings for the proliferation marker Ki67 and for the endothelial junction molecule CD31 revealed a greatly increased number of proliferative endothelial cells in the enlarged blood vessels in UVB irradiated skin, whereas proliferating endothelial cells were rarely detected in control skin. The highest rate of endothelial cell proliferation was observed in the upper dermis of UVB irradiated skin. In non-irradiated epidermis, proliferating epidermal keratinocytes were selectively detected in the basal layer. In contrast, a large number of proliferating keratinocytes was found in the suprabasal layers of the hyperplastic epidermis after UVB irradiation.

A quantitative, computer-assisted morphometric analysis of cutaneous vessel density and size was performed, using CD31-stained tissue sections. Chronic UVB irradiation resulted in a significant ($p < 0.001$) increase in vascular density, as compared with non-irradiated controls. Vessels in UVB irradiated skin were also significantly larger ($p < 0.001$) with a 67% increase in size, leading to a more than 130% increase ($p < 0.001$) in the cutaneous area covered by vessels.

Example 2: Enhanced epidermal VEGF expression after long-term UVB irradiation.

The effect of long-term UVB irradiation on cutaneous VEGF mRNA expression was examined. Using in situ hybridization, it was found that VEGF mRNA expression was potently upregulated in suprabasal epidermal keratinocytes after long-term UVB irradiation, whereas little or no VEGF mRNA expression was detected in the skin of non-UVB irradiated mice.

Example 3: Overexpression of TSP-1 prevents UVB-induced cutaneous damage, wrinkle formation and angiogenesis.

To characterize the biological significance of cutaneous angiogenesis for the effects of long-term UVB irradiation, transgenic mice with skin-specific overexpression of the endogenous angiogenesis inhibitor TSP-1 were subjected to the same UVB irradiation regimen. These mice have been previously characterized in detail and show potent inhibition of induced angiogenesis (24). After 10 weeks of UVB irradiation (cumulative UVB dose of 6.52 J/cm^2), all wildtype mice showed pronounced wrinkle formation on their dorsal skin. In contrast, little or no wrinkle formation was observed in TSP-1 overexpressing transgenic mice. Macroscopically, K14/TSP-1 transgenic mice also showed reduced skin vascularization, as compared with wildtype littermates.

Histological analysis revealed that the UVB-induced thickening of the dermis and the subcutis, but not of the epidermis, was less pronounced in K14/TSP-1 transgenic mice, as compared with wildtype mice. A concomitant reduction of inflammatory cell infiltration was also found and a more regular arrangement and structure of collagen fibers was found in the dermis of K14/TSP-1 transgenic mice as compared to wildtype mice. Moreover, the skin vascular was greatly reduced in K14/TSP-1 transgenic mice. Morphometric analysis of CD31 stained skin sections showed a more than 55% reduction of vessel sizes in TSP-1 transgenic mice ($p < 0.001$) and a significant reduction in the cutaneous area covered by vessels ($p < 0.001$). No significant reduction of the vessel density was detected in TSP-1 transgenic mice. Double immunofluorescent stainings for CD31 and Ki-67 demonstrated a marked reduction in the number of proliferating endothelial cells in the dermis of UVB irradiated TSP-1 transgenic mice, as compared with UVB irradiated wildtype littermates. Moreover, TUNEL assays, combined with CD31 stainings, revealed an increased number of apoptotic endothelial cells in the skin of TSP-1 transgenic mice, as compared with wildtype littermates.

Example 4: Overexpression of TSP-1 prevents UVB-induced MMP-9 activation.

Matrix metalloproteinase-9 (MMP-9) has been implicated in mediating UVB-induced degradation of extracellular matrix components (35), and it has been recently suggested that MMP-9 activity plays a crucial role in angiogenesis by controlling the

bioavailability of VEGF (37). Wildtype and TSP-1 transgenic mice were subjected to a single-dose UVB irradiation (126 mJ/cm^2) of the back skin and MMP-9 activity was determined in skin lysates by gelatin zymography. Single-dose UVB irradiation of wildtype mice resulted in markedly enhanced subcutaneous vascularization after 24 h which was less pronounced in TSP-1 transgenic mice. Gelatin zymography demonstrated equal levels of MMP-9 activity in the normal skin of wildtype and TSP-1 transgenic mice. 24 h after UVB irradiation, however, MMP-9 activity was strongly increased in the skin of wildtype mice but was diminished in TSP-1 transgenic mice.

Example 5: TSP-2 knock out mice show increased wrinkle formation

Long-term UVB irradiation (cumulative UVB dose: 7.23 J/cm^2) produces pronounced wrinkle formation in TSP-2 deficient mice, as compared with wildtype mice. Enlarged cutaneous blood vessels and enhanced vascular branching was seen in TSP-2 deficient mice after chronic UVB irradiation, as compared with wildtype littermates.

Hematoxylin-eosin stains revealed thickening of epidermis and dermis in the skin of TSP-2 deficient mice after long-term UVB irradiation, as compared with wildtype control skin. Trichrome stains demonstrated irregular organization of collagen fibers in the papillary dermis of TSP-2 deficient after chronic UVB irradiation, as compared with wildtype mice. CAE stains revealed increased inflammatory cell infiltration in TSP-2 deficient mice, as compared with wildtype mice.

Immunostains for CD31 revealed more numerous and enlarged blood vessels in the total (dermis + subcutis) skin and in the upper dermis of TSP-2 deficient mice after chronic UVB irradiation, as compared with wildtype littermates. Computer-assisted image analysis of CD31 stained sections revealed a significant increase in vessel size and vessel density in the total skin of TSP-2 deficient mice after chronic UVB irradiation, as compared with wildtype littermates. Similar to vascularization in total skin, the vascularization was also significantly increased in the upper dermis, within a distance of $100 \mu\text{m}$ from the epidermal-dermal border in TSP-2 deficient mice after chronic UVB irradiation.

Double immunofluorescent stains for CD31 and BrdU revealed a pronounced increase in the number of proliferative endothelial cells (arrows) in the upper dermis and the lower dermis of the skin of TSP-2 deficient mice, as compared with wildtype mice.

In situ hybridization for VEGF demonstrated enhanced VEGF mRNA expression in suprabasal keratinocytes of the hyperplastic epidermis in TSP-2 deficient mice after chronic UVB irradiation, as compared with little or no VEGF mRNA expression in wildtype epidermis.

Gelatin-zymography reveals strong induction of MMP-9 activity in the skin of UVB irradiated wildtype mice as compared with non-irradiated wildtype mice. No major differences in MMP-9 activity were detected between TSP-2 deficient mice and wildtype mice after chronic UVB irradiation.

Example 6: Methods and Materials

UVB irradiation regimen.

In a first experiment, 8-week-old female hairless Skh-1 mice (n=7 per group) were exposed to UVB irradiation, using a bank of 4 equally spaced fluorescent lamps (Elder Pharmaceuticals, Bryan, OH) (25). The height of the lamps was adjusted to deliver 0.35 mW/cm² at the dorsal skin surface of the mice. Mice were irradiated with UVB three times weekly for ten weeks, with a starting dose of 0.5 minimal erythema dose (20 mJ/cm²) and gradual increases in increments of 0.5 MED to a maximum dose of 4.5 MED (26). The total cumulative dose of UVB was 5.62 J/cm². No acute sun burn reactions were observed. Control mice were sham-irradiated. In an additional experiment, 8-week-old female K14/TSP-1 transgenic mice (24) or FVB wildtype controls (n=7 per group) were treated with UVB irradiation as described above for a total of 12 weeks (cumulative UVB dose 6.52 J/cm²). After 12 weeks, mice were sacrificed and skin replicas were obtained using silicon rubber (SILFLO; Flexico Developments Ltd, U.K.) as described (27). Back skin samples were either snap-frozen in liquid nitrogen or fixed in 10% formaldehyde as

described (28). All animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Immunohistochemistry for CD31 and computer-assisted morphometric analysis of cutaneous blood vessels.

Immunohistochemical stainings were performed on 7 μm frozen sections as described (24), using a monoclonal rat anti-mouse CD31 antibody (Pharmingen, San Diego, CA). Representative sections were obtained from five UVB irradiated mice and from five age-matched, non-UVB irradiated control mice, and were analyzed using a Nikon E-600 microscope (Nikon; Melville, NY). Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), and morphometric analyses were performed using the IP-LAB software (Scanalytics Inc, Fairfax, VA) as described (24). Three different fields of each section were examined at 60x magnification, and the number of vessels per mm^2 , the average vessel size and the relative area occupied by blood vessels were determined in the dermis, in an area within 100 μm distance from the epidermal-dermal junction. The two-sided unpaired Student's t-test was used to analyze differences in microvessel density and vascular size. In addition, paraffin sections were obtained from the skin of the same mice and routine hematoxylin-eosin, Verhoeff's elastic and Weigert's resorcin fuchsin stains were performed as described (29)

Proliferation and apoptosis assays.

To analyze endothelial cell proliferation, double immunofluorescent stainings for the endothelial cell marker CD31 and the proliferation marker Ki-67 (30, 31) were performed on 7 μm frozen sections, using a monoclonal rat anti-mouse CD31 antibody and a rabbit anti-Ki-67 polyclonal antibody (Novocastra Laboratories, Burlingame, CA). Anti-rat IgG conjugated with FITC and anti-rabbit IgG conjugated with Texas-Red (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies (32). Representative sections were obtained from five mice for each experimental group and were analyzed using a Nikon E-600 microscope. Digital images of CD31 and Ki-67 stains were obtained in identical fields and were combined to reveal

proliferative endothelial cells. Apoptotic endothelial cells were detected by double immunofluorescence, using the Fluorescence-FragEL DNA fragmentation detection kit (Oncogene, Cambridge, MA) and an anti-mouse CD31 antibody together with an anti-rat IgG conjugated with Texas-Red as described (24).

In situ hybridization.

In situ hybridization was performed on paraffin sections as described (19). Briefly, slides were processed through xylene to remove paraffin, then passed sequentially through graded alcohols; 0.2M HCl; Tris/EDTA with 3 µg/ml proteinase K; 0.2% glycine; 4% paraformaldehyde in phosphate-buffered saline pH 7.4; 0.1M triethanolamine containing 1/200 (vol/vol) acetic anhydride; and 2XSSC. Slides were hybridized overnight at 52°C with 35S labeled riboprobes in the following mixture: 0.3M NaCl, 0.01M Tris pH 7.6, 5mM EDTA, 50% formamide, 10% dextran sulfate, 0.1mg/ml yeast tRNA, and 0.01M dithiothreitol. Post-hybridization washes included 2XSSC/50% formamide/10mM dithiothreitol at 65°C and 2XSSC. Slides were then dehydrated through graded alcohol containing 0.3M ammonium acetate, dried, coated with Kodak NTB2 emulsion and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak 19 developer and the slides were counterstained with hematoxylin. Antisense and sense single-stranded 35S-labeled RNA probes for VEGF were prepared from a 393-bp rat VEGF cDNA fragment (12), cloned into pGEM-3Z (Promega).

Gelatin zymography.

The shaved back skin of wildtype FVB mice and transgenic mice (n=4 per group) was exposed to a single dose of UVB irradiation (126 mJ/cm²). After 24 h, mice were sacrificed and back skin samples were excised and homogenized in extraction buffer (0.05M Tris/pH 7.5, 0.2M NaCl, 5mM CaCl₂, 0.1% Triton X-100). After centrifugation, supernatants were collected for gelatin-zymography. Zymography was performed as described (33, 34) with minor modifications. Briefly, skin lysates were resuspended in non-reducing 4X SDS sample buffer (0.5M Tris-HCl/pH 6.8, 0.02% bromophenol blue, 40% (v/v) glycerol, 3% SDS) and were loaded onto SDS polyacrylamide gels containing

0.1% pork skin gelatin (SIGMA). Twenty μ g of each protein lysate were subjected to SDS-PAGE. The gels were incubated with 2.5% Triton X-100 to remove SDS and then overnight with incubation buffer (0.05M Tris-HCl/pH 8.0, 5mM CaCl₂, 5 μ M ZnCl). Gels were then stained with a 0.5% Coomassie brilliant blue R-250/ 30% methanol/ 10% acetic acid solution, followed by destaining using a 30% methanol/ 10% acetic acid solution. MMP-9 activity was detected as a band of 92 kDa molecular weight (35).

1. Kripke, M.L. 1994. Ultraviolet radiation and immunology: something new under the sun-- presidential address. *Cancer Res.* 54:6102-6105.
2. Cox, N.H., B.L. Diffey, and P.M. Farr. 1992. The relationship between chronological age and the erythral response to ultraviolet B radiation. *Br J Dermatol.* 126:315-319.
3. Pearse, A.D., S.A. Gaskell, and R. Marks. 1987. Epidermal changes in human skin following irradiation with either UVB or UVA. *J Invest Dermatol.* 88:83-87.
4. Berton, T.R., D.L. Mitchell, S.M. Fischer, and M.F. Lochniskar. 1997. Epidermal proliferation but not quantity of DNA photodamage is correlated with UV-induced mouse skin carcinogenesis. *J Invest Dermatol.* 109:340-347.
5. Leyden, J.J., G.L. Grove, M.J. Grove, E.G. Thorne, and L. Lufrano. 1989. Treatment of photodamaged facial skin with topical tretinoin. *J Am Acad Dermatol.* 21:638-644.
6. Kligman, A.M. 1989. The treatment of photoaged human skin by topical tretinoin. *Drugs.* 38:1-8.
7. Bielenberg, D.R., C.D. Bucana, R. Sanchez, C.K. Donawho, M.L. Kripke, and I.J. Fidler. 1998. Molecular regulation of UVB-induced cutaneous angiogenesis. *J Invest Dermatol.* 111:864-872.
8. Kramer, M., C. Sachsenmaier, P. Herrlich, and H.J. Rahmsdorf. 1993. UV irradiation-induced interleukin-1 and basic fibroblast growth factor synthesis and release mediate part of the UV response. *J Biol Chem.* 268:6734-6741.

9. Strickland, I., L.E. Rhodes, B.F. Flanagan, and P.S. Friedmann. 1997. TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J Invest Dermatol.* 108:763-768.
10. Yano, K., L.F. Brown, and M. Detmar. 2001. Control of hair growth and follicle size by VEGF-mediated angiogenesis. *J Clin Invest.* 107:409-417.
11. Detmar, M. 1996. Molecular regulation of angiogenesis in the skin. *J Invest. Dermatol.* 106:207-208.
12. Detmar, M., L.F. Brown, K.P. Claffey, K.-T. Yeo, O. Kocher, R.W. Jackman, B. Berse, and H.F. Dvorak. 1994. Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J. Exp. Med.* 180:1141-1146.
13. Brown, L.F., K.T. Yeo, B. Berse, T.K. Yeo, D.R. Senger, H.F. Dvorak, and L. Van De Water. 1992. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J. Exp. Med.* 176:1375-1379.
14. Brown, L.F., T.J. Harrist, K.-T. Yeo, M. Stahle-Backdahl, R.W. Jackman, B. Berse, K. Tognazzi, H.F. Dvorak, and M. Detmar. 1995. Increased expression of vascular permeability factor (vascular endothelial growth factor) in bullous pemphigoid, dermatitis herpetiformis, and erythema multiforme. *J Invest Dermatol.* 104:744-749.
15. Brown, L.F., S.M. Olbricht, B. Berse, R.W. Jackman, G. Matsueda, K.A. Tognazzi, E.J. Manseau, H.F. Dvorak, Van, de, Water, and L. 1995. Overexpression of vascular permeability factor (VPF/VEGF) and its endothelial cell receptors in delayed hypersensitivity skin reactions. *J Immunol.* 154:2801-2807.
16. Detmar, M., L.F. Brown, M.P. Schön, B.M. Elicker, P. Velasco, L. Richard, D. Fukumura, W. Monsky, K.P. Claffey, and R.K. Jain. 1998. Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J. Invest. Dermatol.* 111:1-6.

17. Detmar, M. 2000. The role of VEGF and thrombospondins in skin angiogenesis. *J Dermatol Sci.* 24 Suppl 1:S78-S84.
18. Tolsma, S.S., O.V. Volpert, D.J. Good, W.A. Frazier, P.J. Polverini, and N. Bouck. 1993. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J. Cell Biol.* 122:497-511.
19. Streit, M., P. Velasco, L.F. Brown, M. Skobe, L. Richard, L. Riccardi, J. Lawler, and M. Detmar. 1999. Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am J Pathol.* 155:441-452.
20. Wight, T.N., G.J. Raugi, S.M. Mumby, and P. Bornstein. 1985. Light microscopic immunolocalization of thrombospondin in human tissues. *J. Histochem. Cytochem.* 33:295-302.
21. Bissett, D.L., D.P. Hannon, and T.V. Orr. 1987. An animal model of solar-aged skin: histological, physical, and visible changes in UV-irradiated hairless mouse skin. *Photochem Photobiol.* 46:367-378.
22. Kligman, L.H., C.H. Duo, and A.M. Kligman. 1984. Topical retinoic acid enhances the repair of ultraviolet damaged dermal connective tissue. *Connect Tissue Res.* 12:139-150.
23. Lavker, R.M., and A.M. Kligman. 1988. Chronic heliodermatitis: a morphologic evaluation of chronic actinic dermal damage with emphasis on the role of mast cells. *J Invest Dermatol.* 90:325-330.
24. Streit, M., P. Velasco, L. Riccardi, L. Spencer, L.F. Brown, L. Janes, B. Lange-Asschenfeldt, K. Yano, T. Hawighorst, L. Iruela-Arispe, and M. Detmar. 2000. Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. *EMBO J.* 19:3272-3282.
25. Kochevar, I.E., M. Moran, N. Lyon, T. Flotte, E. Siebert, and R.W. Gange. 1993. Effects of systemic indomethacin, meclizine, and BW755C on chronic ultraviolet B-induced effects in hairless mouse skin. *J Invest Dermatol.* 100:186-193.

26. Kligman, L.H. 1989. The ultraviolet-irradiated hairless mouse: a model for photoaging. *J Am Acad Dermatol.* 21:623-631.
27. Chen, S., I. Kiss, and K.M. Tramposch. 1992. Effects of all-trans retinoic acid on UVB-irradiated and non-irradiated hairless mouse skin. *J Invest Dermatol.* 98:248-254.
28. Streit, M., L. Riccardi, P. Velasco, L.F. Brown, T. Hawighorst, P. Bornstein, and M. Detmar. 1999. Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. *Proc Natl Acad Sci U S A.* 96:14888-14893.
29. Prophet, E., B. Mills, J. Arrington, and L. Sobin. 1992. Laboratory Methods in Histotechnology. American Registry of Pathology, Washington, D. C. 236-237 pp.
30. Key, G., M.H. Becker, B. Baron, M. Duchrow, C. Schluter, H.D. Flad, and J. Gerdes. 1993. New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest.* 68:629-636.
31. Gerdes, J., U. Schwab, H. Lemke, and H. Stein. 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer.* 31:13-20.
32. Skobe, M., P. Rockwell, N. Goldstein, S. Vosseler, and N.E. Fusenig. 1997. Halting angiogenesis suppresses carcinoma cell invasion. *Nat Med.* 3:1222-1227.
33. Delclaux, C., C. Delacourt, M.P. D'Ortho, V. Boyer, C. Lafuma, and A. Harf. 1996. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol.* 14:288-295.
34. Hanemaaijer, R., P. Koolwijk, L. le Clercq, W.J. de Vree, and V.W. van Hinsbergh. 1993. Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. Effects of tumour necrosis factor alpha, interleukin 1 and phorbol ester. *Biochem J.* 296:803-809.

35. Koivukangas, V., M. Kallioinen, H. Autio-Harmanen, and A. Oikarinen. 1994. UV irradiation induces the expression of gelatinases in human skin in vivo. *Acta Derm Venereol.* 74:279-282.
36. Lesnik, R.H., L.H. Kligman, and A.M. Kligman. 1992. Agents that cause enlargement of sebaceous glands in hairless mice. II. Ultraviolet radiation. *Arch Dermatol Res.* 284:106-108.
37. Bergers, G., R. Brekken, G. McMahon, T.H. Vu, T. Itoh, K. Tamaki, K. Tanzawa, P. Thorpe, S. Itohara, Z. Werb, and D. Hanahan. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol.* 2:737-744.
38. Kligman, A.M., G.L. Grove, R. Hirose, and J.J. Leyden. 1986. Topical tretinoin for photoaged skin. *J Am Acad Dermatol.* 15:836-859.
39. Dejana, E., M. Corada, and M.G. Lampugnani. 1995. Endothelial cell-to-cell junctions. *FASEB J.* 9:910-918.
40. Detmar, M., K.-T. Yeo, J.A. Nagy, L. Van De Water, L.F. Brown, B. Berse, B.M. Elicker, S. Ledbetter, and H.F. Dvorak. 1995. Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. *J. Invest. Dermatol.* 105:44-50.
41. Brown, L.F., S.M. Olbricht, B. Berse, R.W. Jackman, G. Matsueda, K.A. Tognazzi, E.J. Manseau, H.F. Dvorak, and L. Van de Water. 1995. Overexpression of vascular permeability factor (VPF/VEGF) and its endothelial cell receptors in delayed hypersensitivity skin reactions. *J Immunol.* 154:2801-2807.
42. Kishimoto, J., R. Ehama, Y. Ge, T. Kobayashi, T. Nishiyama, M. Detmar, and R.E. Burgeson. 2000. In vivo detection of human vascular endothelial growth factor promoter activity in transgenic mouse skin. *Am J Pathol.* 157:103-110.
43. Brauchle, M., J.O. Funk, P. Kind, and S. Werner. 1996. Ultraviolet B and H₂O₂ are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J Biol Chem.* 271:21793-21797.

44. White, F.C., A. Benhacene, J.S. Scheele, and M. Kamps. 1997. VEGF mRNA is stabilized by ras and tyrosine kinase oncogenes, as well as by UV radiation--evidence for divergent stabilization pathways. *Growth Factors*. 14:199-212.
45. Yuan, F., Y. Chen, M. Dellian, N. Safabakhsh, N. Ferrara, and R.K. Jain. 1996. Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. *Proc Natl Acad Sci U S A*. 93:14765-14770.
46. Jimenez, B., O.V. Volpert, S.E. Crawford, M. Febbraio, R.L. Silverstein, and N. Bouck. 2000. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med*. 6:41-48.
47. Yang, Z., D.K. Strickland, and P. Bornstein. 2000. Extracellular MMP2 levels are regulated by the LRP scavenger receptor and thrombospondin 2. *J Biol Chem*. 11:11.
48. Bein, K., and M. Simons. 2000. Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *J Biol Chem*. 275:32167-32173.
49. Taraboletti, G., L. Sonzogni, V. Vergani, G. Hosseini, R. Ceruti, C. Ghilardi, A. Bastone, E. Toschi, P. Borsotti, E. Scanziani, R. Giavazzi, M.S. Pepper, W.G. Stetler-Stevenson, and M.R. Bani. 2000. Posttranscriptional stimulation of endothelial cell matrix metalloproteinases 2 and 1 by endothelioma cells. *Exp Cell Res*. 258:384-394.
50. Kang, S. 1998. Photoaging and tretinoin. *Dermatol Clin*. 16:357-364.
51. Fisher, G.J., Z.Q. Wang, S.C. Datta, J. Varani, S. Kang, and J.J. Voorhees. 1997. Pathophysiology of premature skin aging induced by ultraviolet light. *N Engl J Med*. 337:1419-1428.

Other Embodiments

It is understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the claims.

All patents and references cited herein are incorporated in their entirety by reference. Other embodiments are within the following claims.

We claim:

1. A method of preventing long-term UVB-induced wrinkles in a subject, comprising:
 - identifying a subject in need of wrinkle prevention; and
 - inhibiting angiogenesis in the skin of the subject,
 - thereby preventing long-term UVB-induced wrinkles in a subject.
2. The method of claim 1, wherein angiogenesis is inhibited by increasing TSP-2 or TSP-1 activity in the subject.
3. The method of claim 1, wherein angiogenesis is inhibited by administering to the subject a compound that induces an anti-angiogenesis factor.
4. The method of claim 3, wherein the anti-angiogenesis factor is TSP-1 or TSP-2.
5. A method of preventing or treating a long-term UVB induced wrinkle in a subject, said method comprising administering to the subject a composition comprising an inhibitor of angiogenesis or an agent which induces an inhibitor of angiogenesis in an amount sufficient to reduce or prevent said wrinkle.
6. The method of claim 5, wherein the wrinkle is caused by exposure to natural sunlight.
7. The method of claim 5, wherein the inhibitor of angiogenesis is administered topically.
8. The method of claim 5, wherein the inhibitor of angiogenesis is provided in a sterile composition.

9. The method of claim 5, wherein the inhibitor of angiogenesis is TSP-2 or TSP-1.
10. A composition for preventing or treating long term UVB –induced wrinkles comprising an inhibitor of angiogenesis or an agent that induces an inhibitor of angiogenesis; and a pharmaceutically acceptable carrier.
11. The composition of claim 10, wherein the inhibitor of angiogenesis is TSP-2 or TSP-1.
12. The composition of claim 10, further comprising a cosmetic ingredient
13. The composition of claim 12, wherein the cosmetic ingredient is a fragrance.
14. The composition of claim 12, wherein the cosmetic ingredient is a sunscreen.
15. A method of providing protection against long-term UVB induced wrinkles to a subject, said method comprising:
 - supplying to the subject a composition comprising an inhibitor of angiogenesis or an agent that induces an inhibitor of angiogenesis; and
 - supplying to the subject instructions for using said composition to prevent wrinkles.
16. The method of claim 15, wherein the inhibitor of angiogenesis is TSP-2 or TSP-1.
17. The method of claim 15, wherein said instructions comprise directions to apply the composition to the skin prior to sun exposure.

18. The method of claim 15, wherein the composition further comprises a cosmetic ingredient.

19. A kit for preventing long-term UVB induced wrinkles in a subject, said kit comprising:

a composition comprising an inhibitor of angiogenesis or an agent that induces an inhibitor of angiogenesis; and
instructions for using the composition to prevent wrinkles.

20. The kit of claim 19, wherein said inhibitor of angiogenesis is TSP-1 or TSP-2.

21. The kit of claim 19, wherein said composition further comprises a cosmetic ingredient.

22. The kit of claim 19, wherein said instructions comprise directions to apply said composition to the skin prior to or during sun exposure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/11403

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) :A61K 7/00, 7/42, 7/44, 38/00 US CL :424/59, 60, 400, 401; 514/2 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/59, 60, 400, 401; 514/2 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CAS ONLINE; STN, EAST AND WEST		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,372,234 A (DECKNERS ET AL.) 16 APRIL 2002(16.04.02), See entire document.	1-22
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* "A"	document defining the general state of the art which is not considered to be of particular relevance	* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "E"	earlier document published on or after the international filing date	* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>Felicia D. Roberts</i> SHELLEY A. DODSON Telephone No. (703) 308-1235